Authentication of Traditional Chinese Medicines Radix Aconiti and Radix Aucklandiae by DNA and Chemical Technologies

SHUM Ka Chiu

A Thesis Submitted in Partial Fulfilment
of the Requirements for the Degree of
Master of Philosophy
in
Molecular Biotechnology

The Chinese University of Hong Kong
August 2006

Thesis Assessment Committee

Professor M. M. Y. Waye (Chair)

Professor P. C. Shaw (Thesis Supervisor)

Professor P. P. H. But (Committee Member)

Dr H. Cao (External Examiner)

Acknowledgement

First of all, I would like to express my immense gratitude to my supervisor, Professor P. C. Shaw for his invaluable advice, guidance and patience throughout my study. My gratitude is also extended to Professor P. P. H. But for giving me advice on this research project. I would also like to thank Professor M. M. Y. Waye and Dr H. Cao for sitting in my thesis committee.

I would like to thank Dr H. Cao, Miss L. Cheng, Mr Y. L. Lin and Mr B.Y. Hu for sample collection. I am also indebted to Professor S. M. Liang for his verification of samples.

I would like to extend my gratitude to Mr K. L. Wong, Dr F. Chen, Dr R. W. Jiang, Mr P. M. Hon, Mr Y. M. Chan, Miss W.S. Poon, Mr M. Li, Miss K.Y. Law, Miss H. Lam, Mr C. W. Cheung, Mr Y. H. Chiu, Miss Y. L. Wang, Dr Y. B. Zhang, Dr Y. Sun and other colleagues in the Institute of Chinese Medicine for their generous help and support.

Last but not least, I must express my grateful thanks to my beloved parents, sister and Miss K. W. Chan for their love, patience, support and encouragement.

Abstract

Traditional Chinese Medicine (TCM) has a long history and is widely used in China. Recently, TCM products have become popular in the West. However, problems of adulteration, mis-use or over-dosage can cause potential hazard to the public. Therefore, authentication of TCM is important to ensure the safety and efficacy of TCM. Traditional means of authentication depend mainly on morphological and histological characteristics which could be affected by environmental factors. The experience and judgment of the examiners can also affect the accuracy. Thus, they should be replaced by more objective and reliable authentication methods, including DNA and chemical methods, which are studied in this project.

DNA approaches were used to authenticate Radix Aconiti, which includes the medicinal species *Aconitum carmichaeli* and *A. kusnezoffii*. Both commonly used phylogenetic markers 5S spacer and *psb*A-*trn*H spacer were obtained from different *Aconitum* species. Genomic subtraction was used to screen for sequence markers which allow the discrimination of species listed in *Pharmacopoeia of People's Republic China* and their related species. Marker SSH6 is able to identify *A. kusnezoffii* from other species, while the use of SSH15, 5S spacer and *psb*A-*trn*H spacer can identify the Pharmacopoeia-listed from the unlisted species. Analysis of

SSH45 and 5S spacer suggests that medicinal *Aconitum* species have undergone hybridization. By using SSH15, 5S spacer and *psb*A-*trn*H spacer, we analyzed 17 samples on the market which claimed to be medicinal species, and found that 8 (47%) were not the listed species, showing that the problem of adulteration of *Aconitum* in TCM is a serious problem.

Chemical authentication was used to authenticate Radix Aucklandiae and related medicinal materials. These include *Aucklandia lappa* (Yunmuxiang), *Inula helenium* (Tumuxiang) and *Vladimiria soliei* (Chuanmuxiang). Gas chromatography- mass spectrometry was used to analyze the essential oil contents of the related materials. Results show that the chemical profiles among *A. lappa*, *I. helenium* and *V. soliei* are very different and their applications therefore may not be interchanged. Contents of dehydrocostuslactone and costunolide in *A. lappa* were also determined. We analyzed 27 *A. lappa* samples and 13 (48%) samples were found to be below standard.

Through DNA and chemical authentication, the identity and quality of medicinal materials can be assessed. We also found that adulteration and sub-standard problems of TCM are common. Hence, we suggest that the authenticity and quality

of TCM should be assessed regularly to ensure public safety and health.

摘要

傳統中藥有悠久的歷史、深厚的經驗，在中國及各華人社會得到廣泛的應用。近年，傳統中藥亦漸獲西方社會應用。然而，中藥僞用、誤用均對社會大眾的健康構成威脅。爲保障公眾安全，發展中藥鑑定技術來確保中藥的安全和藥效至爲重要。傳統中藥鑑定方法多是觀察藥材的外內特徵，但由於這些特徵很容易受到外在環境破壞，而且依靠鑑別人員的判斷，結果難免不準確。所以，發展可靠和客觀的鑑定方法是十分必要的。這項研究有關利用分子和化學方法鑑定有毒和珍稀的中藥材。

烏頭類的藥材包括川烏 (*Aconitum carmichaeli*) 和草烏 (*A. kusnezoffii*)。本研究首先測定這兩種藥典品種及其他非藥典品種的核糖體 5S 間區 (nrDNA 5S spacer) 和葉綠體 *psb*A-*trn*H 間區 (*psb*A-*trn*H spacer)。接着，應用基因庫相減 (Genomic subtraction) 的方法把更多的分子標記篩選出來，以便區分藥典和非藥典的烏頭品種。SSH6 可以用來鑑別草烏，而 SSH15、5S 間區和 *psb*A-*trn*H 間區則可以區分藥典和非藥典品種。從分析 SSH45 和 5S 間區，我們更可得知藥用烏頭品種是經過雜交而成。我們以 SSH15、5S 間區和 *psb*A-*trn*H 間區來分析市場上聲稱是藥用烏頭的藥材。在十七個樣本中，我們發現其中八個並非藥典指明的烏頭品種，佔百分之四十七。這反映烏頭類藥材的錯用情況是十分嚴重的。

此外，我們又應用化學方法來鑑定木香類藥材。木香類藥材包括雲木香 (*Aucklandia lappa*)、土木香 (*Inula helenium*) 和川木香 (*Vladimiria soliei*)。木香類藥材中的揮發油被提取、並用氣相色譜—質譜聯用分析儀進行分析。結果確定三種木香類藥材的化學成分十分不同，並不可以交換使用。雲木香成分中去氫木香內酯 (dehydrocostuslactone) 和木香烴內酯 (costunolide) 的含量亦檢測出來。在二十七個從市面上購回來的雲木香樣本中，十三個的質量低於標準，佔百分之四十八。

利用分子鑑定和化學鑑定，我們可以對藥材進行定性和定量的評估。這項研究又發現中藥材有僞品和不合格的問題存在。茲建議有關單位定期對中藥材進行鑑定，以保障公眾安全和健康。

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Abbreviations

A	adenine
AFLP	amplified fragment length polymorphism
AP-1	activating protein-1
BLASTN	nucleotide-nucleotide basic local alignment search tool
bp	base pair
C	cytosine
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	centimeter
CMM(s)	Chinese Medicinal Material(s)
CTAB	cetyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetate
EI^+	positive ion electron impact
eV	electron-volt
G	guanine
g	gram
G+C	guanine+cytosine
GABA	γ-aminobutyric acid
GC-MS	gas chromatography- mass spectrometry
HCA	hierarchical cluster analysis
HPLC	high performance liquid chromatography
in-del	insertion-deletion
iNOS	inducible NO synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
ISSR	inter-simple sequence repeat
ITS	internal transcribed spacer
I-κBα	inhibitory factor-κBα
kb	kilobase pair
KCl	potassium chloride
L	liter
LB	Luria Broth

LBA	LB with ampicillin
M	molar
m	meter
MAPK	mitogen-activated protein kinase
mg	milligram
$MgCl_2$	magnesium chloride
min	minute(s)
ml	milliliter
mM	millimolar
mm	millimeter
MP	maximum parsimony
mRNA	messenger RNA
NaCl	sodium chloride
NaOAc	sodium acetate
NCBI	National Center for Biotechnology Information
NFκB	nuclear transcription factor κB
NH_4OAc	ammonium acetate
NIST	the National Institute of Standards and Technology
NJ	neighbor-joining
NO	nitric oxide
nrDNA	nuclear ribosomal DNA
nrITS	nuclear ribosomal ITS
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
Rf	response factor
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
R.T.	retention time
SDS	sodium dodecyl sulfate
sec	second(s)
T	thymine
TAE	Tris-acetate-EDTA
TCM	Tradition Chinese Medicine
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane

Tris-HCl	Tris-hydrochloride
U	unit
UPGMA	unweighted pair group method with arithmetic mean
UV	ultraviolet
w/v	weight per volume
xg	times gravitational force
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
°C	degree Celsius
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer

Chapter 1. Introduction

Traditional Chinese Medicine (TCM) has been used for over two thousand years in China and the rich and fruitful experience in TCM has been systematically accumulated and well-documented. Recently, TCM products have become popular in the West, in particular the healthcare market. However, reports of adverse effects of TCM have occurred from time to time due to adulterants, mis-use or over-dosage. Safety, efficacy and quality controls are, therefore, a key for the modernization of TCM. To achieve this, authentication of medicinal material is a prerequisite.

1.1 Importance of authentication of Traditional Chinese Medicines

1.1.1 Confusing nomenclatures

Owing to the lack of a unified naming system of medicinal materials in the past, common names are still being used to describe Chinese medicinal materials. These common names vary in different locations or in different literatures, and some common names are shared by different species which may be phylogenetically or pharmacologically unrelated. Most people, who lack specialized training in TCM, may just use the materials according to the common names, and that expose

themselves to potential hazards.

1.1.2 Similar morphologies of different medicinal materials

Most herbal medicinal materials include only parts of a plant. Morphologies of some medicinal materials can look similar, making them difficult to be distinguished from each other. Even worse, some herbal materials are heavily processed before being applied as medicines. During the processing, distinctive characteristics may be diminished or destroyed, and thus hindering the identification.

1.1.3 Toxicities of medicinal materials

Some medicinal materials show a range of toxicity. Mis-use or mis-dosage can cause intoxication in patients. The problem becomes even more serious when the nomenclature issue occurs between a non-toxic one and a toxic one. Cases of TCM intoxication are discussed in later session. To ensure that the medicinal materials being applied are genuine, authentication is necessary.

1.1.4 Conservation of natural products

Herbal materials are natural products with economic value. Yet, over-exploitation of them disturbs the ecosystem. Although the trading of endangered species is

regulated by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), it is difficult to prevent the illegal trading of valuable herbal materials. Authentication techniques can be used to provide evidence for law enforcement.

1.2 TCM listed in the Pharmacopoeia of People's Republic of China

Traditional Chinese Medicine is defined as the medicine which has a long history in Chinese society and those herbal medicines have been literally recorded. The *Pharmacopoeia of People's Republic of China* has listed around five hundred plant and animal species that are commonly used in Traditional Chinese Medicine. Table 1-1 shows the medicinal materials listed in the *Pharmacopoeia of People's Republic of China* (2005) which are related to this study.

Table 1-1. Medicinal materials in the *Pharmacopoeia of People's Republic of China* (2005) which are studied in this project.

Medicinal material	Species	Chinese name
Radix Aconiti and related medicinal materials		
Radix Aconiti	*Aconitum carmichaeli* Debx.	Chuanwu 川烏
Radix Aconiti Preparata	*Aconitum carmichaeli* Debx.	Zhichuanwu 制川烏
Radix Aconiti Lateralis Praeparata	*Aconitum carmichaeli* Debx.	Fuzi 附子
Radix Aconiti Kusnezoffii	*Aconitum kusnezoffii* Reichb.	Caowu 草烏
Radix Aconiti Kusnezoffii Preparata	*Aconitum kusnezoffii* Reichb.	Zhicaowu 制草烏
Folium Aconiti Kusnezoffii	*Aconitum kusnezoffii* Reichb.	Caowuye 草烏葉
Radix Aucklandiae and related medicinal materials		
Radix Aucklandiae	*Aucklandia lappa* Decne.	Muxiang 木香
Radix Inulae	*Inula helenium* L.	Tumuxiang 土木香
Radix Vladimiriae	*Vladimiria souliei* (Franch.) Ling or *Vladimiria souliei* (Franch.) Ling *var. cinerea* Ling	Chuanmuxiang 川木香

1.3 Overview of mis-use and intoxication of TCM

According to a statistic conducted by United Christian Hospital (size of catchment population around 300,000) in Hong Kong from 2001-2003, 75 patients were suspected to be intoxicated by TCM. Eight of them were admitted to intensive care unit as reported by a local newspaper (*Ming Pao*, March 12, 2004).

Reports in recent years from the Department of Health in Hong Kong show that most cases of TCM poisoning were caused by *Aconitum* alkaloids or aristolochic acid (Sin and Chan, 2004; Wong and Chan, 2005). Emergency Room Service of Hospital Authority received cases of TCM intoxication once or twice a week (*Hong Kong Economic Daily*, February 2, 2005).

Besides the high frequency of TCM poisoning, the severity also draws wide-spread attention.

In a study of the accidental herb-induced aconite poisoning in Hong Kong in 1989-91 (Tai *et al.*, 1992), of the 17 cases in the study, 2 died from refractory ventricular fibrillation within 6-hour admission to intensive care unit. Chromatography analysis showed that herb samples from four of those cases contained commonly found *Aconitum* alkaloids – aconitine, mesaconite and hypaconite. Radix Aconiti accounted for 60% of herbal medicine induced accidental poisonings requiring hospital admissions in Hong Kong (Chan, 2002).

A vast number of reports of herbal poisoning in the Mainland China were also recorded (Jin *et al.*, 2005; Li *et al.*, 2000; Liu, 1990; Ruan and Lu, 2000; Zhang and

Yang, 1985; Zhou, 2000). In case of *Aconitum* poisoning, most of the patients required emergency room admission and hospitalization for around 4 days. Although most of the patients could recover after intensive treatment, some might die from excessive intake of unprocessed or under-processed herbal materials, or delayed treatment. Despite the fact that in most cases, *Aconitum* alkaloids could be detected in patients' blood or urine, it was difficult to trace back which herbal material(s) had been used. Official medicinal *Aconitum* species which are listed in the Volume 1 of *Pharmacopoeia of People's Republic of China* include only *Aconitum carmachaeli* and *Aconitum kusnezoffii*. However, over 50 *Aconitum* species are commonly used in Chinese folk medicine, regardless if they are sold as adulterants or used as substitutes.

Recently, the use of Traditional Chinese Medicine has become popular for an alternative effective treatment. Cases of TCM intoxication have also been reported around the world. Moritz and colleagues (2005) reported a case of aconitine poisoning by homemade TCM capsules; Meyer and colleagues (2000) reported a case of nephropathy due to Chinese herbs and suggested the need to regulate herbal medicines.

1.4 Ordinances regulating Chinese medicines as natural products

Development of TCM authentication techniques can help provide forensic evidence. By applying authentication, we can quickly identify the existence of any illegal use of herbal materials. This importance is discussed as follows:

1.4.1 Laws governing Chinese medicine

The regulation of Chinese medicine in Hong Kong is legislated as the Chinese Medicine Ordinance (Cap. 549 of the Laws of Hong Kong) which includes licensing and regulation of Chinese medicine traders and registration of proprietary Chinese medicine. In addition, the Ordinance contains Schedule 1 listing 31 types of toxic or potentially toxic Chinese herbal medicines and Schedule 2 listing 574 types of commonly used Chinese herbal medicines. Those toxic herbal materials listed are in turn regulated by the Import and Export Ordinance (Cap. 60 of the Laws of Hong Kong), which states that the import or export of Schedule 1 herbal materials requires licensing.

Some of the materials like unprocessed Radix Aconiti, Radix Aconiti Kusnezoffii, Radix Aconiti Lateralis, Radix Aconiti Coreani, Radix Aconiti Brachypodi and Radix

Aconiti Szechenyiani are Schedule 1 herbs and they are studied in this project.

1.4.2 Laws governing endangered species

To protect endangered species worldwide, eighty countries agreed on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in mid 1970s. Agreed governments have to, based on the framework of CITES, adopt its domestic legislation to control the trade of endangered species. Up to 2006, more than 160 members have joined the CITES making it one of the largest conservation agreements in the world.

Endangered species are grouped under CITES and listed in three appendices. Appendix 1 includes the species threatened with extinction and no license will be issued; Appendix 2 includes those less threatened and license is required for trading; and Appendix 3 includes those requested by any member. Appendix 1 species which are artificially propagated in registered farms or nurseries are regarded as Appendix 2 species. Up to 2006, some well-known Chinese Medicinal Materials have been listed in the Appendices. These include *Aucklandia lappa* (*Saussurea costus* or *Saussurea lappa*) and *Aloe spp.* in Appendix 1, and *Dendrobium spp.* (except *Dendrobium cruentum* which is in Appendix 1), *Panax ginseng*, *Panax*

quinquefolia and *Podophyllum hexandrum* in Appendix 2.

1.5 Current technologies in the authentication of Traditional Chinese Medicines and their limitations

Traditional means of authentication depend mainly on morphological and histological characteristics. Morphological identification includes the inspection of organoleptic markers such as shape, color, texture and odor of the medicinal materials. Despite its simplicity, its accuracy depends heavily on the examiners' experience and judgment, which is often subjective. It is also difficult to identify herbs which are heavily processed, such as shredded powder or those in extracted products.

Anatomical identification is another means for authentication of herbal materials. Microscopic examination is used to reveal the tissue or cell structures, such as periderm layer, phloem, cambium, xylem and central pith. Distinctive characters may be used to discriminate between genuine species and adulterants. However, factors like age, growth environment and storage condition may alter the structures within the herbal materials. Moreover, closely related species usually have similar

characteristics which prevent the objectiveness of microscopic identification. The experience and judgment of examiner can also alter the accuracy of authentication (Shaw *et al.*, 2002).

Chemical identification makes use of the chemical contents of medicinal materials for authentication. Chromatography including Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography- Mass Spectrometry (GC-MS) is often used to analyze the chemical composition. TLC is simple and can give a quick overview of the chemical profile. However, low resolving power and low repeatability make it difficult to distinguish between closely related species.

HPLC and GC-MS offer a high sensitivity and a very high resolving power which allow the detection of a single compound. Direct analysis of the chemical composition offers a definitive means for quality control of TCM because the pharmacological activities of TCM are brought by its chemical composition. Details of GC-MS are discussed in the later section.

With the advancement of molecular biology, the use of molecular markers plays an

important role in TCM authentication (Shaw *et al.*, 2002). With Polymerase Chain Reaction (PCR), different techniques such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Inter-Simple Sequence Repeat (ISSR) have been developed.

Methods which give different size markers between individuals (revealed by gel electrophoresis) are regarded as a whole genome fingerprint. By using this approach, it is possible to differentiate taxonomic groups at low taxonomic levels as it can capture the small differences between genomes. However, this approach requires high quality genomic DNA. This may not be useful for some CMMs, as they may have undergone a certain degree of processing and a certain length of storage which cause damages to DNA.

In addition to size markers, sequence markers used for phylogenetic purposes can also be used in TCM authentication. This method is further discussed in a later section.

1.6 Historical applications of Radix Aconiti

Aconite has been used for long. Early human recognized the toxicity of aconite and used it as arrow poison in hunting or fighting with enemies (Roberts and Wink, 1998).

The pharmaceutical value of aconite was discovered by Chinese and documented in *Shennong ben cao jing*. The document was written around AD 25-200 in the late East Han Dynasty. Recorded medicinal parts of aconite included the main tuber Wutou and the daughter tuber Tianxiong and Fuzhi. However, the description did not mention the different use of the different *Aconitum* species. In 1578, S.Z. Li divided medicinal aconite into two groups, Chuanwu and Chaowu (Li *et al.*, 1995), which are now known as *Aconitum carmichaeli* and *A. kusnezoffii* respectively.

In the *Pharmacopoeia of People's Republic of China*, only *Aconitum carmichaeli* and *A. kusnezoffii* are listed for medicinal purposes. It is common to use other *Aconitum* species as folk or homeopathic medicines. There are around 35 other *Aconitum* species known to be used (Table 1-2). The use of these substitutes may

be due to the difficulty in identifying *A. carmichaeli* or *A. kusnezoffii* from others, for general public or even experienced practitioners.

Toxicity of aconite was also known to ancient Greeks and Romans. In ancient Rome, aconite was often used in murders and therefore its cultivation was restricted (Rudgley, 1999). Aconite was also used as medicine in the West. Meddygon Myddfai of Wales was among the first ones who published the medicinal use of aconite in the 12th century. In 1762, it was introduced into regular medicine by Baron Störck of Vienna (Osol and Pratt, 1943).

Table 1-2. Common *Aconitum* species used for medicinal purposes in China

Species name	Chinese name 中文名稱	Common name 別名
A. alboviolaceum.	Liangsewutou 兩色烏頭	
A. austroyunnanense	Diannancaowu 滇南草烏	Dacaowu 大草烏 / Xiaoheiniu 小黑牛 / Qixingcaowu 七星草烏
A. barbarum	Niubian 牛扁	
A. brachypodum	Duanbingwutou 短柄烏頭	Xueshangyizhihao 雪上一枝蒿
A. bulleyanum	Dianxicaowu 滇西烏頭	
A. carmichaeli	Chuanwu 川烏	

Table 1-2. Common *Aconitum* species used for medicinal purposes in China

Species name	Chinese name 中文名稱	Common name 別名
(Continued)		
A. chasmanthum	Zhanhuawutou 展花烏頭	
A. changianum	Chawalongwutou 察瓦龍烏頭	Tieluohan 鐵羅漢
A. contortum	Cangshanwutou 蒼山烏頭	Qixingcaowu 七星草烏
A. coreanum	Huanghuawutou 黃花烏頭	Guanbaifu 關白附
A. delavayi	Maershanwutou 馬耳山烏頭	Shuicaowu 水草烏
A. duclouxii	Wujubinchuanwutou 無距賓川烏頭	Baiyao 白葯
A. episcopale	Ziwutou 紫烏頭	Dula 堵喇
A. finetianum	Ganhuanwutou 贛皖烏頭	Poyelian 破叶蓮
A. flavum	Fumaotiebangchui 伏毛鐵棒椎	
A. forrestii	Lijiangwutou 麗江烏頭	Huangcaowu 黃草烏
A. fusungense	Fusongwutou 撫松烏頭	
A. geniculatum	Xibanwutou 膝瓣烏頭 / Dongchuanwutou 東川烏頭	Dacaowu 大草烏
A. gymnandrum	Luruiwutou 露蕊烏頭	
A. hemsleyanum	Guayewutou 瓜葉烏頭	Tengwutou 藤烏頭
A. jaluense	Yaluwutou 鴨綠烏頭	
A. karakolicum	Duogenwutou 多根烏頭	

Table 1-2. Common *Aconitum* species used for medicinal purposes in China

Species name	Chinese name 中文名稱	Common name 別名
(Continued)		
A. kirinense	Jilinwutou 吉林烏頭	
A. kongboense	Gongbuwutou 工布烏頭	
A. kusnezoffii	Beiwutou 北烏頭	Caowu 草烏
A. liangshanicum	Liangshanwutou 涼山烏頭	Xueshangyizhihao 雪上一枝蒿 / Xuewu 雪烏
A. liljestrandii	Gonggawutou 貢嘎烏頭	
A. nagarum	Baoshanwutou 保山烏頭 / Xuanweiwutou 宣威烏頭	Xiaobaicheng 小白撐 / Xueshangyizhihao 雪上一枝蒿
A. naviculare	Chuankuiwutou 船盔烏頭	Bangga 榜嘎
A. paniculigerum	Yuanzhuiwutou 圓錐烏頭	
A. pendulum	Tiebangchui 鐵棒棰	
A. polyschistum	Duoliewutou 多裂烏頭	Daxueshangyizhihao 大雪上一枝蒿
A. pulchellum	Meiliwutou 美麗烏頭	Xiaobaicheng 小白撐
A. racemulosum	Yanwutou 岩烏頭	
A. richardsonianum	Fumaozhixuwutou 伏毛直序烏頭	Xiyecaowu 細葉烏頭
A. rotundifolium	Yuanyewutao 圓葉烏頭	
A. scaposum	Huatingwutou 花葶烏頭	Moqi 墨七

Table 1-2. Common *Aconitum* species used for medicinal purposes in China

Species name	Chinese name 中文名稱	Common name 別名
(Continued)		
A. sessiliflorum	Suogengwutou 縮梗烏頭	Xueshangyizhihao 雪上一枝蒿
A. sinomontanum	Gaowutou 高烏頭	Mabuqi 麻布七
A. stapfianum	Yulongwutou 玉龍烏頭	Heixinjie 黑心解
A. sungpanense	Songpanwutou 松潘烏頭	Huoyanzi 火焰子
A. taipaicum	Taibaiwutou 太白烏頭	Jinniuqi 金牛七
A. transsectum	Zhiyuanwutou 直緣烏頭	
A. umbrosum	Caodiwutou 草地烏頭	Heidajiao 黑大艽
A. vilmorinianum	Huangcaowu 黃草烏	Dacaowu 大草烏
A. volubile	Manwutou 蔓烏頭	

References: Editorial Board of *Chinese Materia Medica,* State Administration of Traditional Chinese Medicine of People's Republic of China (1998a); Li *et al.*, 1995; Yang *et al.*, 1993

1.7 Modern applications of Radix Aconiti

Owing to high toxicity of raw *Aconitum* tuber, most applications of *Aconitum* require processing by boiling the tubers for 1 to 2 hours. Modern pharmacological studies have provided evidence for a wide range of activities from *Aconitum* or its alkaloid components, including analgesic, anti-inflammation, anti-tumor, anti-rheumatic,

strengthening the heart, lowering the blood pressure and lowering the blood glucose.

Reports show that *Aconitum* can be used in modern clinical treatments: (1) as analgesic in surgical operations. (2) as external analgesic in treatment of shoulder periarthritis, strain of lumbar muscles, sciatica and arthralgia. (3) as anti-tumor and analgesic in cancer (Hu, 2000).

The lateral root of *Aconitum carmichaeli* is also used in enhancing sex ability in male. For example, a formulation including processed *Aconitum* lateral root can replenish the function of kidney and the sex ability as described in old Chinese documents (Wang, 2000).

1.8 Research on Radix Aconiti and its chemical components

1.8.1 Chemistry

Modern researches reveal that most of the pharmacological activities of *Aconitum* are brought by *Aconitum* alkaloids. Alkaloids are one of the most diverse groups of plant secondary metabolites. In the Genius *Aconitum*, two major types of pharmacologically active alkaloids can be found: diterpene alkaloids and quinoline

alkaloids (Li, 1995). Diterpene alkaloids are subdivided into two classes depending on the number of carbon atoms in the skeleton structure, namely C_{19}-skeleton and C_{20}-skeleton (Pelletier and Keith, 1970a). Interestingly, the pharmacologically-active alkaloids are at the same time highly toxic when they are in higher concentrations. In order to apply Radix Aconiti safely as medicine, quality control, especially in determining the amount of alkaloids, is important.

There is a long history of *Aconitum* alkaloid research studies. Aconitine was first discovered from *Aconitum napellus* in 1833; and later the crystal of aconitine was isolated in 1860 (Felter and Lloyd, 1898). However, analysis of these naturally-occurring chemical has remained difficult since related alkaloids often form mixtures that could not be separated (Stern, 1954). Up to now more than 450 alkaloids have been isolated from the genus *Aconitum* and new structures continue to be found (Zhao *et al.*, 2003).

Representatives of C_{19}-diterpene alkaloids are aconitine, mesaconitine, hypaconitine and yunaconitine, which are common and abundant in many *Aconitum* species. This type of alkaloids is more toxic than the other types. C_{20}-diterpene alkaloids are another group of chemicals which can also be found in *Aconitum*. Examples are

songorine and napelline. Compared to diterpene alkaloids, quinoline alkaloids are also found in some of the *Aconitum* species in lower amount, such as magnoflorine and higenamine (Li *et al.*, 1995).

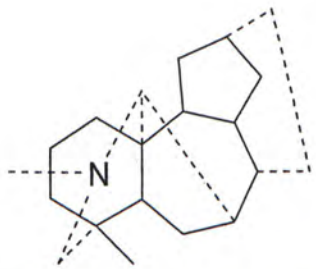

Figure 1-1. The basic skeleton of common C_{19}-diterpene alkaloids found in *Aconitum* species. The structure is a hexacyclic skeleton which is comprised of one seven-membered, three six-membered and two five-membered rings (Pelletier and Keith, 1970b)

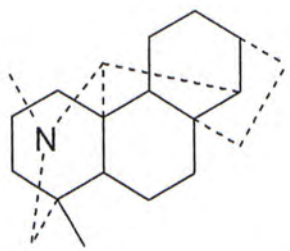

Figure 1-2. The basic skeleton of common C_{20}-diterpene alkaloids found in *Aconitum* species. The structure is a hexacyclic skeleton which is comprised of four six-membered and two five-membered rings (Pelletier and Keith, 1970c)

1.8.2 Pharmacology

Recent pharmacological studies reveal a wide range of activities from *Aconitum* alkaloids. One of the most distinctive effects is the analgesic and antinociceptive activities which was proven in writhing-assay and tail flick test in mice (Ameri,

1998). Experiments showed that activation of inhibitory noradrenergic neurons via β-adrenoceptor was a possible mechanism. Lappaconitine showed lower toxicity compared with aconitine and mesaconititne, and lower analgesic activity than mesaconitine.

Anti-inflammatory effects are another activity from *Aconitum* alkaloids. Both mesaconitine and 3-acetylaconitine were shown anti-inflammatory effects in sham-operated and adrenalectomized mice. However, the mechanism of anti-inflammation remains unclear: a group reported that mesaconitine could not inhibit the synthesis of prostaglandins (Ameri, 1998), while another group supports that inhibition of prostaglandin E synthesis by total Aconitum alkaloids resulted in the anti-inflammatory effects (Shi *et al.*, 1990).

Higenamine is another *Aconitum* alkaloid which can inhibit inflammation. Higenamine inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) mRNA in RAW 264.7 cells in a concentration-dependent manner (Kang *et al.*, 1999). They suggested that this *Aconitum* alkaloid inhibited iNOS expression by inhibiting nuclear factor κB (NFκB). Recent findings concluded that NF-κB induces four classes of genes including immunity, anti-apoptosis, proliferation and

negative feedback (Karin *et al.*, 2002), implying that this alkaloid might also have anti-tumor and anti-cancer activities.

Antiepileptiform activity of *Aconitum* alkaloid was also observed *in vitro* at rat hippocampal slice model. *Aconitum* alkaloids found to have this activity include lappaconitine, 6-benzoylheteratsine, 1-benzoylnapelline and mesaconitine (Ameri, 1998). Studies showed that lappaconitine inhibited selectively excessive neuronal activity and blocked the generation and spread of aberrant activity. On the other hand, mesaconitine attenuated both stimulus-triggered and spontaneous epileptiform activities. Evidence showed that the inhibitory action of mesaconitine involved α-adrenoceptor.

Aconitum alkaloids also show activities in the cardiovascular system. It is interesting that different alkaloids gave opposite effects: aconitine, 3-acetylaconitine and mesaconitine induced tacharrhythmia while lappaconitine, N-deacetyllappaconitine heteratisine and napelline gave antiarrhythmic effects (Ameri, 1998). As suggested by the author, lappaconitine and N-deacetyllappaconitine reduced blood pressure by the activation of cardiac reflex receptors. The vasorelaxing effect of mesaconitine on rat small gastric arteries was

also reported (Mitamura *et al.*, 2002).

1.8.3 Molecular interaction

Recent research studies reveal various molecular interactions between *Aconitum* alkaloids and ion channels or receptors. Aconitine is a persistent activator of sodium ion channels. It activates sodium channels by blocking its inactivation and that shifts the voltage-dependence of the channel activation to a more negative membrane potential (Ameri, 1998). In contrast to aconitine, lappaconitine irreversibly blocks sodium channels in heart tissue (Wright, 2001). In addition to the interaction to sodium channels, another *Aconitum* alkaloid songorine was found to be a $GABA_A$ receptor antagonist in the rat brain (Zhao *et al.*, 2003).

Although there is a wide range of studies about *Aconitum* and its alkaloid contents, until now, there is still a lack of research studies on individual *Aconitum* species. The alkaloid contents of each *Aconitum* species are still unclear, and it is expected that there is a range of combinations of alkaloids among different species. What makes the situation more complicated is that different *Aconitum* alkaloids can show contrasting activities. Therefore, it can be dangerous to apply *Aconitum* as medicine if the species are wrongly identified. Before a comprehensive

understanding of the alkaloid contents, authentication can provide a safety and quality assurance measure.

1.9 Brief review on the systematics and phylogeny of *Aconitum*

Aconitum is a genus in tribe Delphineae under the family Ranunculaceae. The genus *Aconitum* consists of around 400 species and is distributed in Eurasia, North America and North Africa.

The record of genus *Aconitum* can be traced back to 1753 when Linnaeus recorded five *Aconitum* species in *Species Plantarum*. Since then, the systematic of this group has been controversial. Currently the most widely accepted system is to divide the genus into three subgenera: Subgen. *Lycoctonum*, Subgen. *Aconitum* and Subgen. *Gymnaconitum* (Tamura, 1995; Wang, 1979). Subgen. *Gymnaconitum* is monotypic and it contains only one annual species, *A. gymnandrum*. It is generally considered the most advanced species in the genus. Subgen. *Aconitum* is characterized by its biennial paired tuberous root, while Subgen. *Lycoctonum* has perennial rhizomes.

Aconitum is a complicated group in which the grouping of subgenera, series and species is difficult to handle. The morphological variations between groups are usually continuous, especially under the subgenus *Aconitum*. Moreover, most of the studies of this group are localized rather than globalized. Due to the lack of a comprehensive study, there is still no consensus on the systematics of this group.

Outline of the systematic arrangement of the genus *Aconitum* according to the *Flora of China* is shown as follows and only the *Aconitum* species, which are related to this study, are shown.

Subgen. *Lycoctonum*

- Sect. Paraconitum
- Sect. Fletcherum

Subgen. *Aconitum*

- Sect. Sinaconitum
- Sect. Aconitum
  - Ser. Tangutica
  - Ser. Bullatifolia
    - *Aconitum nagarum*
  - Ser. Brunnea
  - Ser. Stylosa
  - Ser. Racemulosa

Ser. Volubilia

Aconitum hemsleyanum

Aconitum vilmorinianum

Ser. Inflata

Aconitum carmichaeli

Aconitum kusnezoffii

Ser. Ambigus

Ser. Grandituberosa

Ser. Brachypoda

Aconitum coreanum

Subgen. *Gymnaconitum*

1.10 Historical applications of Radix Aucklandiae and related materials

The use of *Aucklandia lappa* as medicine was also recorded in *Shennong ben cao jing*, together with *V. souliei* and *I. helenium* under the same name Muxiang. There was no discrimination of the pharmacological effects of the three medicinal species, and they were commonly called Qingmuxiang.

Aucklandia lappa is also used in India as herbal healing and called Kushtha in Indian. The Indian *A. lappa* was imported to Guangdong, China by trading. At that time *A. lappa* acquired the name Guangmuxiang. But there was still no concrete

discrimination of the three medicinal species.

At the same time, other medicinal species *Aristolochia debilis* and *Aristolochia contorta* which belong to Aristolochiaceae and have different medicinal effects also shared the name Qingmuxing. In 1578 when it was the Ming dynasty in China, S.Z. Li noticed the naming problem of Muxiang. To elucidate that, Li grouped the *Aristolochia* materials to Qingmuxiang, and renamed *Aucklandia lappa* from Qingmuxiang to Guangmuxiang. Recently in China, *A. lappa* is cultivated in large amount for medicinal purpose in Yunnan, China, and it is now officially called Yunmuxiang. *Vladimiria soliei* also acquires the present name Chuanmuxiang as it is largely cultivated in Sichuan province in China.

Aucklandia lappa is also applied for various purposes outside China. In the Alpine region, *A. lappa* is used as an insecticide which is powered and sprinkled over crops. It is also used for toothache and against the heart diseases of cattle. The roots are externally used for the treatment of maggot-infested wound (Alpine Medicinal Herbs & Rural Welfare Organization, 2006). More recently it has also been used in Western aromatherapy.

1.11 Modern applications of Radix Aucklandiae and related material

Aucklandia lappa is widely applied in Chinese medicine or medicinal products. It is mainly used to treat problems in the alimentary canal. *A. lappa* is also used to treat diarrhea in children (Zhou, 2001). In the treatment of peptic ulcer, the combined use of proprietary Chinese medicine and Western medicine showed a higher efficacy than using Western medicine alone (Yin, 2002). Chronic gastritis could also be treated by a formulation including *Aucklandia lappa*. Research shows the use of Chinese medicine in treatment of chronic gastritis provided a higher efficacy than ordinary Western medicine (Liu *et al.*, 2004). Moreover, another proprietary Chinese medicine containing *Aucklandia lappa* could be used in treatment of cancer. The use of *Aucklandia lappa* lessened the symptoms caused by cancer and recovered the functions of the alimentary canal (Huang, 2001).

In addition to the treatment of the alimentary canal, *Aucklandia lappa* was used to treat coronary heart disease (Hu and Yang, 2000). The material was also used to treat acute pancreatitis, acute cholecystitis and pulmonary infection (Tian *et al.*, 2000).

1.12 Research on Aucklandiae and related material and their chemical components

1.12.1 Chemistry

Essential oil is one of the most remarkable secondary metabolites found in the root of *Aucklandia lappa* and related medicinal species. Research on *Aucklandia lappa* essential oil made progress in early twentieth century when costusic acid, costuslactone and dihydrocostuslactone were first purified by Semmler and Feldstein in 1914 (Parry, 1922). Recent findings reported that the root of *Aucklandia lappa* consists of a rich content of sesquiterpene lactones. Dehydrocostuslactone and costunolide are the two most dominating sesquiterpene lactones which consist of up to 50% of total essential oil content (Editorial Board of *Chinese Materia Medica,* State Administration of Traditional Chinese Medicine of People's Republic of China 1998b; Singh *et al.*, 1992).

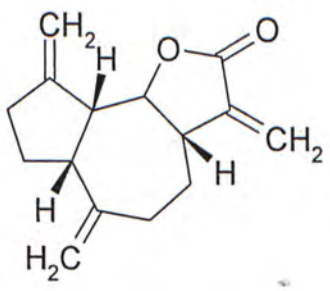

Figure 1-3. The molecular structure of dehydrocostuslactone

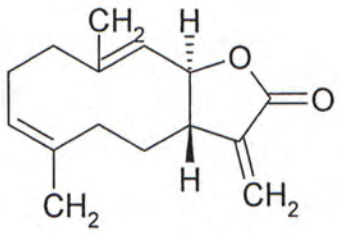

Figure 1-4. The molecular structure of costunolide

1.12.2 Pharmacology

Modern pharmacological studies on *Aucklandia lappa* essential oil revealed that the *Aucklandia* lactones could relax smooth muscles in digestive system, bronchus (Gupta and Ghatak, 1967) and blood vessels. Moreover, research studies in cancer also revealed the activities of *Aucklandia* lactones in the immune system and in cancer cells, including anti-tumor and anti-angiogenesis, and this suggested that these lactones could be used in the treatment of various cancers.

These lactones have various activities along the digestive system. They retarded gastric emptying (Matsuda *et al.*, 1999). Also, the *Aucklandia* extracts could first increase a little and then decrease the rate and power of peristalsis of isolated rat ileum (Wang *et al.*, 1982).

These lactones showed contrasting activities to the heart and blood vessels in low or high concentrations. When the concentration was low, these lactones could increase heart rate as shown in frog and dog models, cause dilation in rat ear blood vessels and increase peripheral blood flow (Editorial Board of *Chinese Materia Medica,* State Administration of Traditional Chinese Medicine of People's Republic of China, 1998b). However, in high concentration, the lactones reduced heart rate, and caused contraction in blood vessels.

Recent research studies in *Aucklandia* lactones concentrate on its pharmacological activities towards tumors and cancer because of their activities to block activation of nuclear transcription factor κB (NFκB). Both dehydrocostuslactone (Oh *et al.*, 2004) and costunolide (Koo *et al.*, 2001) enhanced tumor necrosis factor-α-induced apoptosis in human leukemia cells by preventing degradation of I-κBα. Costunolide inhibited interleukin-1β expression by down-regulating AP-1 and MAPK activities probably also through the NFκB pathway (Kang *et al.*, 2004).

Costunolide also showed antiangiogenic effect that it inhibited the vascular endothelial growth factor (Jeonga *et al.*, 2002). Costunolide also inhibited the telomerase activity in human breast carcinoma cells (Choi *et al.*, 2005).

Costunolide inhibited cancer growth also by interacting with microtubules as shown in MCF-7 cells and affecting the formation of cytoskeleton (Bocca *et al.*, 2004).

As *Aucklandia lappa* contains a rich content of sesquiterpene lactones, it can be a very effective medicine in treating cancers. However, as the concentration of the lactones content determines their pharmacological activities, a quantitative way of authentication is important.

1.13 Brief review on the systematics and phylogeny of *Aucklandia* and related medicinal species

Aucklandia lappa (*Saussurea lappa*) and related medicinal species, *Vladimiria soliei* (*Dolomiaea soliei*) and *Inula helenium*, belong to the family Asteraceae (Compositae). Both *Aucklandia* and *Vladimiria* are grouped into the subtribe Carduinae under tribe Cardueae. While Carduinae is a classic case of a plesiomorphic and paraphyletic assemblage (Bremer, 1994), it is suggested that *Aucklandia* and *Jurinea* should constitute a separate group in this subtribe (Dittrich, 1977). *Inula helenium* is grouped into Tribe Inuleae under another subfamily called Asteroideae.

The following is a simplified scheme of the systematics of selected Asteraceae groups according to Bremer, 1994.

Family Asteraceae

Sub-family Barnadesioideae

Sub-family Cichorioideae

Tribe Mutisieae

Tribe Cardueae

Subtribe Carduinae

Genus *Vladimiria*

Vladimiria souliei

Vladimiria berardioides

Genus *Aucklandia*

Aucklandia lappa

Tribe Lactuceae

Tribe Vernonieae

Tribe Liabeae

Tribe Arctoteae

Sub-family Asteroideae

Tribe Inuleae

Genus *Inula*

Inula helenium

Inula racemosa

Tribe Plucheeae

Tribe Gnaphalieae

Tribe Calenduleae

Tribe Astereae

Tribe Anthemideae

Tribe Senecioneae

Tribe Helenieae

Tribe Heliantheae

Tribe Eupatorieae

1.14 Authentication by DNA sequencing

1.14.1 Introduction

DNA sequencing offers a definitive means for identifying medicinal materials as most of them are originated from living organisms. This technique has been applied on the authentication of TCM in various species (Lau *et al.*, 2000; Ngan *et al.*, 1999; Wong *et al.*, 2004).

DNA as a genetic material is unique to each individual. By comparing the sequence of the suspected samples and that of authentic samples, we can quickly figure out the identity of the samples.

Generally, only the representative regions in the genome are sequenced in TCM

authentication and these DNA regions are often used in phylogenetic analysis. They are called sequence markers.

There is a wide range of commonly used markers which can differentiate organisms at different taxonomic levels, because different regions in the genome evolve in different rates. In fact, during evolution, variations including transversion, transition, insertion or deletion occur in the genome, and different regions will have different rates of variation. In general, relationships at low taxonomic level can be inferred by fast evolving DNA regions, and vice versa. Table 1-3 shows the approximate taxonomic level of utility of commonly used DNA markers for differentiation of plants.

Table 1-3. Approximate taxonomic level of utility of various DNA markers for differentiation of plants

Marker	Approximate taxonomic level
Nuclear genome	
ITS	Species-Genus
5S rDNA spacer	Population-Species-Genus
5.8S rDNA	Family-Order-Class-Phylum
26S rDNA	Family-Order-Class-Phylum
18S rDNA	Family-Order-Class-Phylum
Chloroplast genome	
*trn*L-*trn*F spacer	Species-Genus-Family
*trn*L intron	Species-Genus-Family
*atp*B-*rbc*L intergenic region	Species-Genus
*mat*K	Species-Genus-Family-Order
*ndh*F	Genus-Family-Order-Subclass
*rbc*L	Genus-Family-Order-Subclass
*psb*A-*trn*H spacer	Species-Genus
Mitochrondrial genome	
*cox*I	Subclass-Phylum

Reference: Soltis and Soltis, 1998

Although there are three genomes in plant, mitochondrial genome is less used for inferring relationship due to its rapid changes in genome structure and size (Judd *et al.*, 1998; Soltis and Soltis, 1998). Markers used for inferring plant phylogeny are commonly found in the nuclear genome and chloroplast genome. These markers are also gaining attention in species identification. Several criteria should be considered for choosing the suitable markers for TCM authentication.

1.14.2 Criteria of sequence markers

Taxonomic level: Markers should be chosen at differentiable taxonomic level between the genuine TCM and its adulterants.

Copy number: Copy number refers to the number of copies of a specific DNA region within a cell. A high copy number means more regions of the same type can be found for a given amount of sample. Markers of higher copy number are generally preferred as this can give a higher successful rate for obtaining the sequence.

Concerted evolution of multiple copies of specific markers: Concerted evolution refers to the homogenization of multiple copies of a specific marker to the same sequence. When concerted evolution of markers affects less on some markers, the markers give more variable sequences which make it difficult for authentication. 5S spacer is a well-known example for un-concerted evolution.

Length: Shorter markers are preferred. Many of the CMMs available on the market are processed to different degrees. Such process may degrade the DNA. It is generally difficult to obtain sequence information from fragmented DNA.

Shorter DNA will have a higher chance to escape from the fragmentation.

Existence of contamination: Contamination may be introduced to CMMs during processing or storage. Fungal contamination is commonly found. DNA markers from plants may also be found in fungi. nrITS is one of the examples. Sequences from contaminants will interfere with the results. As chloroplast exists only in plants, using markers from chloroplast can generally prevent the problem of fungal contamination. However, this does not exclude the utility of nuclear markers for plant materials.

Searching for suitable markers which can meet the above criteria is therefore crucial in TCM authentication.

1.14.3 Model used to process polymorphism in DNA sequences

Genetic events like insertion, deletion, transition and transversion cause polymorphisms in DNA. Although in-dels can be used to differentiate species, they are not used for phylogram contruction as the algorithm does not consider in-dels. The DNA substitution model, Kimura 2-parameter (Kimura, 1980), is used to calculate the pairwise distance between two sequences and construct phylograms

using UPGMA and NJ, based on the transitions and transversions. In Kimura 2-parameter, every base (A,T,C,G) is considered having equal frequency to occur, and the rate of substitution does not vary among sites. The distance scores given by transition are higher than that by transversion as it assumes that transition is more frequent than transversion. Phylograms constructed by MP is by finding the most parsimony (least substitution) phylogram among the sequences.

1.15 Screening for novel markers

1.15.1 Reason for screening novel markers

Not all species can be authenticated by existing DNA markers. Sometimes the markers are too conserved or too diverse across closely related species. In order to apply sequencing for authentication under such circumstances, we have to screen for novel markers which allow us to identify the medicinal species from the adulterants accurately.

In this study, a technique called subtractive hybridization is employed for screening for novel markers. By subtracting one genome from another, we can identify sequences that are different from one another.

1.15.2 Basic principle

The basic principle is as follows. The genomic DNA sample that contains sequence of interest is called tester while the reference is called driver. The tester and driver DNA are digested with a four-base-cutting restriction enzyme. The tester DNA is then divided into two portions. Each portion is ligated with different adaptors. Two rounds of hybridization are then performed.

In the first round of hybridization, excess driver DNA is added to each ligated tester DNA. The mixtures are heated and allowed to re-anneal. Common DNA strands between tester and driver will anneal to each other, and the remaining unique tester DNA will be in single or double stranded form.

In the second round of hybridization, the two mixtures from the first hybridization are mixed together. Single stranded tester DNA will anneal to each other, forming a double strand DNA with each end ligated to different adaptors.

The tester-specific DNA is then amplified by PCR in two rounds. In the second round, nested primers are used to further enrich the tester-specific sequences.

After that, the tester-specific sequences are inserted into cloning vectors to generate a subtraction library between the tester and driver. They will be sequenced and primers will be designed to check for their ability to identify specific medicinal species from their adulterants.

1.16 Introduction to gas chromatography- mass spectrometry

Gas chromatography- mass spectrometry (GC-MS) is the combination of two powerful analytical techniques. Gas chromatography separates a mixture of chemicals, and at the same time, mass spectrometry breaks a chemical into fragments which provide clues of its structure. By using GC-MS, we can analyze the content even from trace amount of samples. The advantages of fastness, accuracy and sensitivity make it a popular technique for the authentication of medicinal materials. However, as gas chromatography requires a gas phase separation, only chemicals which sublime or boil at the temperature range can be analyzed. That limits the use of GC-MS to mainly volatile chemicals.

The extract of most traditional medicinal material consists of a mixture of chemicals. The composition of the chemicals may be different due to its origin, growth

condition and storage method. Different organs may also contain a slightly different composition. As the chemical composition is directly associated with the pharmacological effects, GC-MS can provide a direct assessment of TCM quality.

1.16.1 Basic principles and components of GC-MS

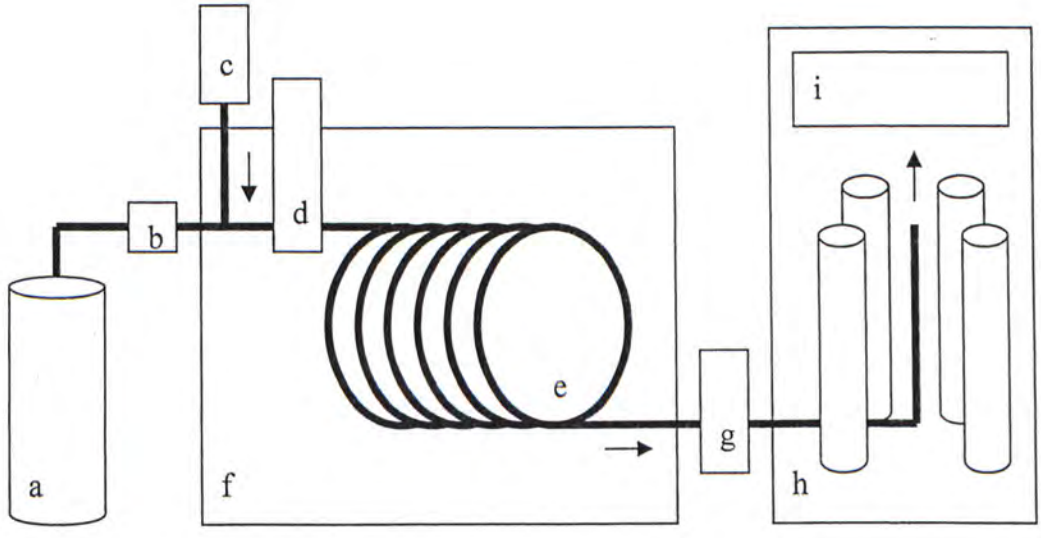

Figure 1-5. Overview of a typical GC-MS system (not in-scale). (a) Carrier gas (b) Flow control valve (c) Sample injector (d) Variable split (e) Capillary column (f) Oven (g) Detector (h) Mass spectrometer (i) Mass detector. Arrows represent the flow of samples.

Figure 1-5 shows a typical layout of a GC-MS system. The sample is injected into the column by sample injector. The mobile phase is provided by the carrier gas whose velocity is controlled by flow control valve. Inert gas such as hydrogen, helium or nitrogen is chosen so as to provide an inert environment for the sample.

The inert gas provides the mobile phase and the column provides the stationary phase. There is a wide variety of columns available on the market with different polarities, internal diameters and lengths. In the course of chromatography, the oven controls the temperature of the column, thus in turn controls the separation. Generally, lower temperature gives a better resolution but requires longer analysis time. Instead of using a constant temperature, the use of temperature programming is common for separation of sample mixture with wide boiling points (McNair and Miller, 1998). After the separation, the effluents are detected by a detector for data collection.

Effluent, after passing through the detector, will pass on to the mass spectrometer in which every component gives a mass spectrum.

1.16.2 Advantages and limitations of GC-MS

GC-MS is a very powerful analytic tool and it has the following advantages and limitations (McNair and Miller, 1998): First, GC-MS analysis is rapid and with robotic injectors, unattended operation is possible. Also, the resolution and sensitivity is high, and even small amounts of samples can be analyzed. Moreover, the detector responses to the effluents quantitatively. Therefore, by setting up

standardization, the amount of effluents can be determined by GC-MS. Furthermore, by mass spectrometry, the identity of each chemical can be inferred. However, only the volatile composition can be detected as GC-MS requires a gas phase separation. Thermally labile samples cannot be analyzed directly as they will be broken down at a high temperature along the column. GC-MS is also a piece of expensive equipment but the running cost is low in the long run.

1.16.3 Usage of GC-MS on natural product analysis

The advancement of GC-MS and chemometric analysis enables researchers to reveal the complex chemical composition of plants. Brooker and Lassak (1981) used GC-MS to distinguish between *Eucalyptus ovata* and *E. brookerana*. Since then, different species such as *Thymus*, *Pinus*, *Juniperus* and *Mentha* were also analyzed by different groups (Hibbert, 1997). GC-MS was also used for analyzing *Aucklandia* and related species such as the determination on the essential oil constituents of *Aucklandia lappa* (Qiu *et al.*, 2001) and the testing of the different parameters for better resolution of GC-MS on *Vladimiria souliei* (Zhang and Gao, 2001).

1.16.4 Chemometric analysis

Automation allows GC-MS data to be collected in digital format which is excellent for analysis such as mass spectra library search, chromatography normalization and chemometric analysis. Chemometric analysis combines computational and statistical methods to extract useful information, and it is important for comparing natural products. Hierarchical Cluster analysis (HCA) is one of the most important analyses for taxonomical purposes. This method involves the calculation of pairwise distances or similarities between two individuals, and then the linkage between individuals. HCA can give an easily understandable form of result in form of a dendrogram, which is a tree diagram showing the relationship among all the samples. There are different distance formulae and linkage rules. Hibbert recommended the use of Euclidean distance with unweighted pair group average linking for taxonomical purposes (Hibbert, 1997). However this does not limit the use of other methods, and both squared Euclidean distance and cosine of vectors of variables will be presented in this study for comparison.

In HCA, it can be imagined that each sample is represented by a coordinate in which each parameter is presented by a dimension in a multi-dimensional coordinate system. HCA is a method of grouping samples which are closer to each other in the

coordinate system.

Squared Euclidean distance and cosine of vectors of variables are two of the methods for calculating distances between samples. The first one calculates the direct distance between two coordinates. Its formula is given as

$$d_{ij} = \sum_{k=1}^{k=n} (x_{i,k} - x_{j,k})^2$$

where d_{ij} is the distance between sample i and j in an n-dimensional space. The later one regards each coordinate as a vector and calculates the cosine value between two vectors. It gives values of similarity and its formula is given as

$$s_{ij} = \frac{\sum_{k=1}^{k=n} (x_{i,n} x_{j,n})}{\sqrt{\left(\sum_{k=1}^{k=n} x_{i,n}^2\right)\left(\sum_{k=1}^{k=n} x_{j,n}^2\right)}}$$

where s_{ij} is the similarity between sample i and j in an n-dimensional space.

1.17 Objectives

To promote public health, protect public safety and help modernization of Traditional Chinese Medicine, authentication of Chinese Medicinal Materials is essential. In this study, different techniques for authentication are demonstrated and several objectives are to be achieved:

1. To identify *Aconitum carmichaeli*, *Aconitum kusnezoffii* and other *Aconitum* species by DNA sequencing;
2. To screen for novel sequence markers for authentication of *Aconitum* and related species;
3. To identify *Aucklandia lappa*, *Vladimiria soliei*, *Inula helenium* and related species by gas chromatography- mass spectrometry;
4. To assess the quality of Radix Aucklandiae by quantitatively detect its chemical components.

Chapter 2. Materials and Methods

2.1 Plant samples

2.1.1 Samples of *Aconitum*

Various samples of *Aconitum* were collected and their species identities were confirmed: 10 samples of *A. carmichaeli*, 8 samples of *A. kusnezoffii*, 1 sample of *A. coreanum*, 1 samples of *A. hemsleyanum*, 2 samples of *A. nagarum* and 4 samples of *A. vilmorinianum*. Samples on the market were also collected. Details of individual samples are listed in Table 2-1. All voucher samples have been deposited in Institute of Chinese Medicine, the Chinese University of Hong Kong.

Table 2-1. A list of samples of *Aconitum* species used in this study

Code	Source	Form	Collector
Species name: *Aconitum carmichaeli*			
Name in Chinese: Chuanwu 川烏			
AC2	Sichuan, China	Medicinal Material	H. Cao
ACQ	Royal Botanic Gardens, Kew	Extracted DNA	---
ACS	Royal Botanic Gardens, Kew	Seed	---
AC19	Xian Botanical Garden	Leaf specimen	H. Cao
AC21	Institute of Botany, the Chinese Academy of Sciences	Leaf specimen	H. Cao
AC23	Kunming Institute of Botany	Leaf specimen	H. Cao
AC24	Sichuan, China	Leaf specimen	H. Cao
AC25	Kunming Institute of Botany	Leaf specimen	H. Cao

Table 2-1. A list of samples of *Aconitum* species used in this study

Code	Source	Form	Collector
(Continued)			
ACfz1	Sichuan, China	Medicinal material from lateral root	H. Cao
ACfz2	Chongqing, China	Medicinal material from lateral root	H. Cao
Species name: *Aconitum kusnezoffii*			
Name in Chinese: Caowu 草烏			
AK1	Heilongjiang, China	Medicinal Material	H. Cao
AK2	Heilongjiang, China	Medicinal Material	H. Cao
AK6c	Guangxi, China	Medicinal Material	H. Cao
AK9a	Sichuan, China	Medicinal Material	H. Cao
AK10	Sichuan, China	Medicinal Material	H. Cao
AK19	Xian Botanical Garden	Leaf specimen	H. Cao
AK21	Institute of Botany, the Chinese Academy of Sciences	Leaf specimen	H. Cao
AKfz2	Guangxi, China	Medicinal material from lateral root	H. Cao
Species name: *Aconitum coreanum*			
Name in Chinese: Huanghuawutou 黃花烏頭			
AKo1	Heilongjiang, China	Medicinal material	H. Cao
Species name: *Aconitum hemsleyanum*			
Name in Chinese: Guayewutou 瓜葉烏頭			
AH1	Yunnan, China	Medicinal material	H. Cao
Species name: *Aconitum nagarum*			
Name in Chinese: Baoshanwutou 保山烏頭			
AN1	Yunnan, China	Medicinal material	H. Cao
AN2	Yunnan, China	Medicinal material	H. Cao
Species name: *Aconitum vilmorinianum*			
Name in Chinese: Huangcaowu 黃草烏			
AV1	Yunnan, China	Medicinal material	H. Cao
AV2	Yunnan, China	Medicinal material	H. Cao

Table 2-1. A list of samples of *Aconitum* species used in this study

Code	Source	Form	Collector
(Continued)			
AV3	Yunnan, China	Medicinal material	H. Cao
AV4	Yunnan, China	Medicinal material	H. Cao
Species name: *Aconitum sp.*			
Name in Chinese: Wutou 烏頭			
AC1	Guangxi, China	Medicinal material claimed as *A. carmichaeli*	H. Cao
AC3	Sichuan, China	Medicinal material claimed as *A. carmichaeli*	H. Cao
AC4	Guizhou, China	Medicinal material claimed as *A. carmichaeli*	H. Cao
AC5	Chongqing, China	Medicinal material claimed as *A. carmichaeli*	H. Cao
AC7	Neimenggu, China	Medicinal material claimed as *A. carmichaeli*	H. Cao
AC11	Hong Kong, China	Medicinal material claimed as *A. carmichaeli*	L.Cheng
AC12	Hong Kong, China	Medicinal material claimed as *A. carmichaeli*	L.Cheng
AC13	Hong Kong, China	Processed medicinal material claimed as *A. carmichaeli*	L.Cheng
AC14	Hong Kong, China	Processed medicinal material claimed as *A. carmichaeli*	L.Cheng
AC15	Hong Kong, China	Processed medicinal material claimed as *A. carmichaeli*	L.Cheng
AC16	Hong Kong, China	Processed medicinal material claimed as *A. carmichaeli*	L.Cheng
AC17	Hong Kong, China	Processed medicinal material claimed as *A. carmichaeli*	L.Cheng
AC18	Hong Kong, China	Processed medicinal material claimed as *A. carmichaeli*	L.Cheng
AC20	Not known	Processed medicinal material claimed as *A. carmichaeli*	H. Cao
ACfz3	Sichuan, China	Medicinal material from lateral root claimed as *A. carmichaeli*	H. Cao

Table 2-1. A list of samples of *Aconitum* species used in this study

Code	Source	Form	Collector
(Continued)			
AK3	Sichuan, China	Medicinal material claimed as *A. kusnezoffii*	H. Cao
AK4	Guizhou, China	Medicinal material claimed as *A. kusnezoffii*	H. Cao
AK5	Liaoning, China	Medicinal material claimed as *A. kusnezoffii*	H. Cao
AK7	Sichuan, China	Medicinal material claimed as *A. kusnezoffii*	H. Cao
AK8	Guizhou, China	Medicinal material claimed as *A. kusnezoffii*	H. Cao
AK9b	Yunnan, China	Medicinal material claimed as *A. kusnezoffii*	H. Cao
AK11	Hong Kong, China	Medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK12	Hong Kong, China	Medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK13	Hong Kong, China	Processed medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK14	Hong Kong, China	Processed medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK15	Hong Kong, China	Processed medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK16	Hong Kong, China	Processed medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK17	Hong Kong, China	Processed medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK18	Hong Kong, China	Processed medicinal material claimed as *A. kusnezoffii*	L.Cheng
AK20	Not known	Processed medicinal material claimed as *A. kusnezoffii*	H. Cao
AK23	Yunnan, China	Leaf specimen claimed as *A. kusnezoffii*	H. Cao
AK24	Not known	Processed medicinal material claimed as *A. kusnezoffii*	H. Cao

Table 2-1. A list of samples of *Aconitum* species used in this study

Code	Source	Form	Collector
(Continued)			
AKfz1	Heilongjiang, China	Medicinal material from lateral root claimed as *A. kusnezoffii*	H. Cao
AS1	Not known	Medicinal material	H. Cao
AS2	Not known	Medicinal material	H. Cao
AS3	Yunnan, China	Medicinal material	H. Cao
AS4	Yunnan, China	Medicinal material	H. Cao
ASfz1	Not known	Medicinal material from lateral root	H. Cao
ASfz2	Not known	Medicinal material from lateral root	H. Cao
ASfz3	Yunnan, China	Medicinal material from lateral root	H. Cao

2.1.2 Samples of *Aucklandia* and related species

Various samples were collected and their species identities were confirmed: 35 samples of *Aucklandia lappa*, 20 samples of *Inula helenium*, 5 samples of *Inula racemosa*, 20 samples of *Vladimiria souliei*, 20 samples of *Vladimiria souliei* var. *cinerea* and 5 samples of *Vladimiria berardioides*. Details of individual samples are listed Table 2-2. All voucher samples have been deposited in Institute of Chinese Medicine, the Chinese University of Hong Kong.

Table 2-2. A list of samples of *Aucklandia lappa* and related species used in this study

Code	Source	Form	Collector
Species name: *Aucklandia lappa*			
Name in Chinese: Yunmuxiang 雲木香			
AL-1	Sichuan, China	Medicinal material	H. Cao
AL-2	Sichuan, China	Medicinal material	H. Cao
AL-3	Sichuan, China	Medicinal material	H. Cao
AL-4	Sichuan, China	Medicinal material	H. Cao
AL-5	Sichuan, China	Medicinal material	H. Cao
AL-6	Sichuan, China	Medicinal material	H. Cao
AL-7	Sichuan, China	Medicinal material	H. Cao
AL-8	Sichuan, China	Medicinal material	H. Cao
AL-9	Guizhou, China	Medicinal material	H. Cao
AL-10	Guizhou, China	Medicinal material	H. Cao
AL-11	Chongqing, China	Medicinal material	H. Cao
AL-12	Chongqing, China	Medicinal material	H. Cao
AL-13	Chongqing, China	Medicinal material	H. Cao
AL-14	Chongqing, China	Medicinal material	H. Cao
AL-15	Guizhou, China	Medicinal material	H. Cao
AL-16	Guizhou, China	Medicinal material	H. Cao
AL-18	Yunnan, China	Medicinal material	H. Cao
AL-19	Yunnan, China	Medicinal material	H. Cao
AL-20	Yunnan, China	Medicinal material	H. Cao
AL-21	Yunnan, China	Medicinal material	H. Cao
AL-22	Yunnan, China	Medicinal material	H. Cao
AL-23	Yunnan, China	Medicinal material	H. Cao
AL-25	Yunnan, China	Medicinal material	H. Cao
AL-25a	Guangdong, China	Medicinal material	H. Cao
AL-26	Guangxi, China	Medicinal material	H. Cao
AL-27	Yunnan, China	Medicinal material	H. Cao
AL-28	Guangxi, China	Medicinal material	H. Cao
AL-31	Yunnan, China	Medicinal material	H. Cao
AL-32	Yunnan, China	Medicinal material	H. Cao
AL-34	Hong Kong, China	Medicinal material	H. Cao
AL-37	Hubei, China	Medicinal material	H. Cao
AL-38	Hebei, China	Medicinal material	H. Cao

Table 2-2. A list of samples of *Aucklandia lappa* and related species used in this study

Code	Source	Form	Collector
(Continued)			
AL-39	Guangdong, China	Medicinal material	H. Cao
024-089-1# AL-40	Nation Institute for the Control of Pharmaceutical and Biological Products, China	Medicinal material	---
024-089-2# AL-41	Nation Institute for the Control of Pharmaceutical and Biological Products, China	Medicinal material	---
Species name: *Inula helenium*			
Name in Chinese: Tumuxiang 土木香			
IH01	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH02	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH03	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH04	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH05	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH06	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH07	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH08	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH09	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH10	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH11	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH12	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin

Table 2-2. A list of samples of *Aucklandia lappa* and related species used in this study

Code	Source	Form	Collector
(Continued)			
IH13	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH14	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH15	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH16	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH17	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH18	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH19	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
IH20	Hebei, China	Medicinal material and leaf specimen	Y.L. Lin
Species name: *Inula racemosa*			
Name in Chinese: Zongzhuangtumuxiang 總狀土木香			
IR01	Qinghai, China	Medicinal material and leaf specimen	Y.L. Lin
IR02	Qinghai, China	Medicinal material and leaf specimen	Y.L. Lin
IR03	Qinghai, China	Medicinal material and leaf specimen	Y.L. Lin
IR04	Qinghai, China	Medicinal material and leaf specimen	Y.L. Lin
IR05	Qinghai, China	Medicinal material and leaf specimen	Y.L. Lin
Species name: *Vladimiria berardioides*			
Name in Chinese: Houyechuanmuxiang 厚葉川木香			
VB01	Yunnan, China	Medicinal material and leaf specimen	Y.L. Lin

Table 2-2. A list of samples of *Aucklandia lappa* and related species used in this study

Code	Source	Form	Collector
(Continued)			
VB02	Yunnan, China	Medicinal material and leaf specimen	Y.L. Lin
VB03	Yunnan, China	Medicinal material and leaf specimen	Y.L. Lin
VB04	Yunnan, China	Medicinal material and leaf specimen	Y.L. Lin
VB05	Yunnan, China	Medicinal material and leaf specimen	Y.L. Lin
Species name: *Vladimiria souliei*			
Name in Chinese: Chuanmuxiang 川木香			
VS01	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu and Y.L. Lin
VS02	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS03	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS04	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS05	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS06	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS07	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS08	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS09	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS10	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS11	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu

Table 2-2. A list of samples of *Aucklandia lappa* and related species used in this study

Code	Source	Form	Collector
(Continued)			
VS12	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS13	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS14	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS15	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS16	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS17	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS18	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS19	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VS20	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
Species name: *Vladimiria souliei* var. *cinerea*			
Name in Chinese: Huimaochuanmuxiang 灰毛川木香			
VSV01	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV02	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV03	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV04	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV05	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV06	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu

Table 2-2. A list of samples of *Aucklandia lappa* and related species used in this study

Code	Source	Form	Collector
(Continued)			
VSV07	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu and Y.L. Lin
VSV08	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu and Y.L. Lin
VSV09	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu and Y.L. Lin
VSV10	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV11	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV12	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV13	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV14	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV15	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV16	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV17	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV18	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV19	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu
VSV20	Sichuan, China	Medicinal material and leaf specimen	B.Y. Hu

2.2 DNA extraction method

2.2.1 Reagents

SDS Extract Buffer

200mM Tris-HCl

200mM NaCl

25mM EDTA

0.5% SDS

pH 8.0

Protease K

2X CTAB Buffer

2%w/v CTAB

100mM Tris-HCl

20mM EDTA

1.4M NaCl

1% PVP

pH 8.0

CTAB precipitation buffer

50mM Tris-HCl

10mM EDTA

1%w/v CTAB

24:1 chloroform:isoamyl alcohol

1M NaCl

100% ethanol

70% ethanol

Autoclaved MilliQ H_2O

2.2.2 Methods

The extraction method which was modified from Draper and Scott (1988) and Kang *et al.* (1998) was based on cetyl-triethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) respectively. Surface contamination was removed by rinsing with distilled water and ethanol alternatively. Around 0.25g plant or medicinal material was weighed and cut by sterilized scissors. The sample was then incubated with 500µl SDS Extraction Buffer with 50µg protease K in a microcentrifuge tube at 37°C for one hour. Further grinding was performed by sterilized scissors until the smallest possible pieces were obtained. The mixture was further incubated for one hour. Then 500µl 2X CTAB buffer was added to the mixture. After a 10min 12,000xg centrifugation, 600µl supernatant was transferred

to a new microcentrifuge tube. Then 600µl 24:1 chloroform:isoamyl alcohol was added to the mixture. The microcentrifuge tube was inverted 5 times to allow a brief mixing. A 10min 12,000xg centrifugation was performed and the aqueous portion was transferred to a new tube. Another 600µl 24:1 chloroform:isoamyl alcohol was added to the aqueous portion; the tube was inverted 5 times; a 10min 12,000xg centrifugation was done and the aqueous portion was transferred to a new tube. Then 600µl CTAB precipitation buffer was added to the aqueous portion and the mixture was allowed to stand at room temperature for 30min. After that, a 30min 12,000xg centrifugation was done and the supernatant was discarded. Next, 400µl 1M NaCl was added to dissolve the pellet and 800µl 100% ethanol was then added. The tube was then put in -20°C for 30min. A 30min 12,000xg centrifugation was done and the supernatant was discarded; 1ml 70% ethanol was added; a 10min 12,000xg centrifugation was done and the supernatant was discarded. The pellet was dried in a 37°C oven for 2 hours. Finally, 20µl MilliQ water was added to dissolve the DNA pellet.

2.3 Chemical extraction methods

Total essential oil extraction was modified from the method described in Appendix X D, Volume 1, *Pharmacopoeia of the People's Republic of China*. First, 50g of medicinal material was ground into powder and added to the round-bottom flask (Figure 2-1:A), with 500ml distilled water. After a brief mixing, the round-bottom flask, essential oil distiller (Figure 2-1:B) and reflux condenser (Figure 2-1:C) were connected accordingly. With the valve of the distiller closed, distilled water was added through the reflux condenser to the distiller until water flowed out to the flask. Electric mantle was then used to heat up the mixture to a mild boiling for 5 hours. An oily layer would be found at the top of the water level in the distiller. After the apparatus colded down, water was drained down and the oil was collected by a dropper. Anhydrous sodium sulfate was added to absorb water content in the oil extract.

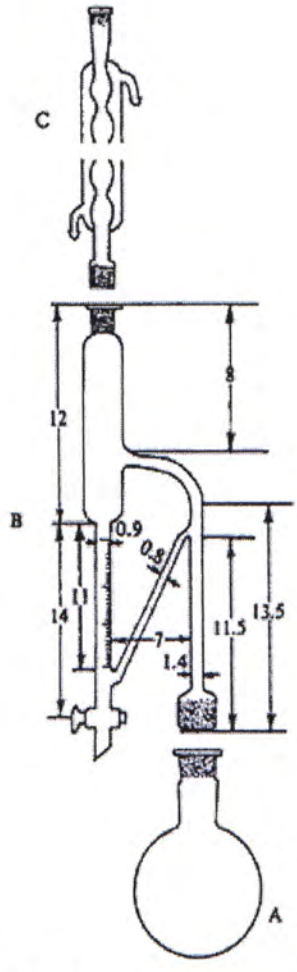

Figure 2-1. Essential oil extraction apparatus. A: 1000ml round-bottom flask. B: essential oil distiller. C: reflux condenser.

2.4 Chemical standard extraction and purification method

Two kilogram *Aucklandia lappa* was cut into small pieces of around 1cm diameter and refluxed in 5L absolute ethanol in patches. The ethanol extract was then combined, vacuum-dried and resuspended in 500ml water. The suspension was then undergone three partitions of hexane, chloroform and ethyl acetate accordingly. For each solvent, partition was done twice and 250ml of solvent was used each time.

Silica gel column chromatography was further performed to purify each chemical component. For each chemical component, serial dilution was made and analyzed by GC-MS for a standard curve.

2.5 DNA sequencing

2.5.1 Reagents

10X PCR buffer

10mM Tris-HCl

50mM KCl

25mM $MgCl_2$

0.001% gelatin

pH 8.3

2.5mM dNTP

Taq polymerase

Autoclaved MilliQ

1X TAE buffer

40mM Tris-HCl

40mM Acetic acid

2mM EDTA

Autoclaved

Ethidium Bromide

LB

10%w/v tryptone

5%w/v yeast extract

0.085M NaCl

pH 7.4

Autoclaved

LBA

LB

50 µg/ml ampicillin

LBA agar

LB

15% Agar

Autoclaved then 50µg/ml ampicillin

2.5.2 Methods

Polymerase chain reaction (PCR) was carried out to amplify a region to be sequenced. Each PCR reaction mixture was prepared in the following composition: 2.5µl 10X PCR buffer, 2µl 2.5mM dNTP, primer pair of 1µl 25µM each (listed in Table 2-4) and 1µl 1U *Taq* polymerase. The reaction mixture was added up to 25µl with appropriate amount of DNA template and undergone the thermal cycles as shown in Table 2-3.

Table 2-3. Thermal cycles used in PCR of DNA sequence markers

Step	Temperature	Length	Purpose
1.	94°C	5min	Initial denaturation
2.	94°C	1min	Denaturation
3.	56°C	45sec	Annealing
4.	72°C	1min15sec	Extension
5.	35 cycles of step 2 to 4		
6.	72°C	5min	Final extension
7.	4°C		Storage

Table 2-4. Primers used in DNA sequencing and their sequences

Primer name	Primer sequence
Flanking region: 5S spacer	
S1	GGA TCC GTG CTT GGG CGA GAG TAG TA
AS1	GGA TCC TTA GTG CTG GTA TGA TCG CA
Flanking region: *psb*A-*trn*H spacer	
psbA	GTT ATG CAT GAA CGT AAT GCT C
trnH2R	CGC GCA TGG TGG ATT CAC AAT CC

Agarose gel electrophoresis was performed to check the specificity of the reaction. The gel was cast using 1.5% w/v agarose in 1X TAE buffer with 0.5μg/ml ethidium bromide. The PCR mixture was mixed with 6X dye in 6:1 ratio and loaded into the gel. Electrophoresis was done at 7V/cm for 30min. After that, the gel was examined under UV illumination in BIORAD Gel Documentation System 1000. Specific PCR products would be shown in the form of sharp bands, and the bands were cut for DNA recovery.

DNA recovery was performed by using the commercial kit Gel-M™ Gel Extraction System from Viogene-Biotek Corp. The procedure was performed as stated in the protocol supplied. 500μl GEX buffer was added to the piece of gel containing specific PCR product, and incubated in 65°C for 15 min or until the gel was dissolved thoroughly. The whole mixture was transferred to the supplied cartridge and centrifuged at 12,000xg for 1min. The flow-through was discarded. 500μl WF buffer was then added to the cartridge and centrifuged at 12,000xg for 1min. The flow-through was discarded. Next, 700μl WS was added to the cartridge and centrifuged at 12,000xg for 1min. After the flow-through was discarded, the cartridge was centrifuged further at 12,000xg for 3min to remove any residue. The cartridge was placed inside a new recovery microcentrifuge tube and 30μl

70°C-prewarmed MilliQ water was added to the cartridge for DNA elution. After a 12,000xg 1min centrifugation, the DNA elution was collected and stored for further experiments.

Eluted DNA would be ligated to cloning vector for cloning and sequencing. Ligation was performed by using the commercial kit pGEM®–T Easy Vector System I from Promega Corp. The procedure was modified from the protocol supplied. The ligation reaction mixture was prepared as follows: 2.5μl 2X rapid ligation buffer, 0.5μl T4 DNA ligase, 0.25μl pGEM®–T Easy vector with 1.75μl eluted DNA. The mixture was incubated at 25°C for 2 hours.

Ligation product was transformed to *E. coli* DH5α competent cells and in this part, aseptic technique was adopted. First, 200μl competent cells were thawed in ice bath. Then the ligation product was added to the cell and kept on ice for 10min. A 42°C 2min heat-shock was performed and after that the cells was placed back on ice for 2min. 100μl LB (prewarmed to 37°C) was added to the cells for 30min recovery. The cells were spread on LBA agar plate evenly with 5μl 0.4M IPTG and 20μl 5% X-gal, and incubated at 37°C for 16 hours.

A single cell should grow to a single colony on the LBA agar plate after approximately 16 hours. One white colony was picked for *psb*A-*trn*H while eight white colonies were picked for 5S spacer. Each picked colony was inoculated in 1ml LB with 50μg/ml ampicillin and incubated at 37°C for 16 hours. The culture was then centrifuged at 12,000xg for 2min. The supernatant was discarded and the cell pellet was kept for plasmid miniprep.

Plasmid miniprep was performed by using the commercial kit Mini-M® Plasmid DNA Extraction System from Viogene-Biotek Corp, and the procedure was performed as stated on the protocol supplied. The cell pellet was suspended in 250μl MX1. 250μl MX2 and 350μl MX3 was added to the mixture accordingly with mixing. The mixture was centrifuged at 12,000xg for 10min. The supernatant was transferred to the supplied cartridge and after a 1min 12,000xg centrifugation; the flow-through was discarded. 500μl WF was added to the cartridge and centrifuged at 12,000xg for 1min. The flow-through was discarded. Next, 700μl WS was added to the cartridge and centrifuged at 12,000xg for 1min. After the flow-through was discarded, the cartridge was centrifuged further at 12,000xg for 3min to remove any residue. The cartridge was placed inside a new recovery microcentrifuge tube and 30μl 70°C-prewarmed MilliQ water was added to

the cartridge for plasmid elution.

The commercial kit BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems was used for cycle sequencing. The procedure was modified from the protocol. 4μl MilliQ, 2μl reaction mix, 1μl 5X sequencing buffer, 1μl 1.6M sequencing primer and 2μl plasmid eluent was mixed. For each eluent, sequencing was performed twice, each using T7 promoter primer (5'-TAA TAC GAC TCA CTA TAG GG-3') or SP6 primer (5'-ATT TAG GTG ACA CTA TAG AAT-3'). The mixture was then undergone the following thermal cycles:

Table 2-5. Thermal cycles for cycle sequencing

Step	Temperature	Length	Purpose
1.	95°C	30 sec	Denature
2.	50°C	25 sec	Annealing
3.	60°C	5 min	Extension
4.	25 cycles of step 1-3		

After cycle sequencing, a brief purification was done. 1μl 3M NaOAc of pH 5.3 and 25μl 100% ethanol were added to the mixture and incubated at -20°C for 30min. The mixture was then centrifuged at 12,000xg for 30min and the supernatant was discarded. 200μl 70% ethanol was added and centrifuged at 12,000xg for 5min. Supernatant was again discarded and the pellet was dried in a 37°C oven. The pellet

was dissolved in 12μl Hi-Di formamide from Applied Biosystems and transferred to 96-well sequencing plate for sequence analysis. The solution was denatured at 95°C for 3min and kept on ice immediately. The samples were then analyzed by an ABI 3100 Genetic Analyzer from Applied Biosystems.

Sequence analysis was accomplished by using bioinformatics software: Chromas Lite 2.0 from Technelysium Pty Ltd for sequence quality check; ClustalW for aligning multiple sequences (Chenna *et al.*, 2003); BioEdit 7.0.1 from Tom Hall Isis Pharmaceuticals Inc for sequence alignment editing (Hall, 1999); and MEGA 3.1 for phylogenetic and molecular evolutionary analysis (Kumar *et al.*, 2004).

2.6 Genomic subtraction

Genomic subtraction was performed by using the commercial kit PCR-Select™ Bacterial Genome Subtraction Kit from Clontech. The procedures were modified from the user manual supplied.

Total DNA extracts from sample *A. carmichaeli* ACfz2 and *A. kusnezoffii* AK2 were selected as the tester and driver respectively. Both the tester and driver were

digested into small fragments by the restriction enzyme *Rsa*I. The digestion mixture was set as follows: 5μl 10X *Rsa*I restriction buffer, 1.5μl (15U) *Rsa*I, 2μg DNA and addition of MilliQ to make the total volume up to 50μl. The mixture was incubated at 37°C for 16 hours. 2.5μl 0.2M EDTA was added to stop the reaction. 50μl 25:24:1 phenol:chloroform:isoamyl alcohol was added. The mixture was mixed vigorously and then centrifuged at 12,000xg for 10min. The top aqueous layer was transferred to a new microcentrifuge tube and mixed with 50μl 24:1 chloroform:isoamyl alcohol. After a 12,000xg 10min centrifugation, the top aqueous layer was transferred to a new tube and 25μl 4M NH_4OAc and 187.5μl 95% ethanol was added. After a thorough mixing, the mixture was centrifuged at 12,000xg for 20min. The supernatant was removed and 200μl 80% ethanol was added and another 12,000xg 5min centrifugation was done. The supernatant was removed and the pellet was dried in a 37°C oven. 6.5μl MilliQ water was added.

Tester DNA would be further processed by the following procedures: 1.2μl digested DNA was diluted by adding 1.8μl MilliQ water. Two ligations were made: one with adaptor 1 and the other with adaptor 2R. The ligation mixture was set as follows: 1μl diluted tester DNA, 2μl 10μM adaptor, 2μl 5X ligation buffer, 1μl 400U T4 DNA ligase and 4μl MilliQ water. The mixture was incubated at 16°C overnight.

1μl 0.2M EDTA was added and the sample was heat to 72°C for 5min to stop the reaction.

Diluted driver and ligated testers would undergo hybridization. Mixture 1 contained 2μl diluted driver DNA, 1μl adaptor-1 ligated tester DNA and 1μl 4X hybridization buffer. Mixture 2 contained adaptor-2R ligated tester DNA instead. The two mixtures were incubated at 98°C for 90sec then 63°C for 90min. Meanwhile, a mixture of 1μl driver DNA, 0.5μl 4X hybridization buffer and 0.5μl MilliQ was incubated at 98°C for 90sec. The three mixtures were then mixed and incubated at 63°C overnight. 200μl dilution buffer was added and incubated at 63°C for 7min.

1μl aliquot from the diluted subtracted was amplified in the following mixture: 19.5μl MilliQ, 2.5μl 10X PCR reaction buffer, 0.5μl 10mM dNTP, 1μl 10μM PCR Primer 1 and 0.5μl *Taq* polymerase. The thermal cycle was performed as follows:

Table 2-6. Thermal cycles used in pre-amplification of genomic subtraction

Step	Temperature	Length	Purpose
1.	72°C	2min	Repair of protruding ends
2.	94°C	30sec	Denature
3.	66°C	30sec	Annealing
4.	72°C	1min30sec	Extension
5.	25 cycles from step 2 to 4		

1μl PCR product was then diluted by 39μl MilliQ and 1μl of the dilution was amplified for another round of PCR in the following mixture: 18.5μl MilliQ, 2.5μl 10X PCR reaction buffer, 1μl 10μM Nested Primer 1, 1μl 10μM Nested Primer 2R, 0.5μl 10mM dNTP and 0.5μl *Taq* polymerase. The thermal cycle was performed as follows:

Table 2-7. Thermal cycles used in nested-PCR in genomic subtraction

Step	Temperature	Length	Purpose
1.	94°C	30sec	Denature
2.	68°C	30sec	Annealing
3.	72°C	1min30sec	Extension
4.	12 cycles from step 1 to 3		

The PCR product was then ligated to the vector from the pGEM®–T Easy Vector System I, transformed to *E. coli* DH5α and miniprep as described above. A subtraction library was then established.

2.7 Search for species-specific markers from the subtraction library

After each clone was sequenced, primer pairs were designed to amplify the preliminary markers. Each marker was amplified by its primer pair for each *Aconitum* sample shown previously. Sequencing of the markers adopted the previous method. After obtaining the sequence information, markers from different samples were aligned and the feasibility of being species-specific markers was determined.

2.8 Gas chromatography- mass spectrometry

Extracted essential oil samples were diluted 100-1000 times by hexane, and filtered by 0.45μm Millipore filtration unit. 5μl sample was injected into the GC-MS system at a time. Agilent 6890 series GC system with a 5973 mass selective detector was used and GC-MS system was set as the following condition: The column was a HP-5MS fused silica capillary column (crosslinked (5%-Phenyl)-methylpolysiloxane, 30m×0.25mm I.D., 0.25μm film thickness). The column temperature was initially maintained at 80°C for 2 minutes, then risen from 80°C to 220°C at the rate of 2°C/min, from 220°C to 260°C at the rate of 20°C/min;

and finally at 260°C for 5 minutes. The inlet temperature was set to 230°C; the carrier gas used was helium with a flow rate 1ml/min; ionization source heater temperature was set to 280°C; the positive ion electron impact (EI^+) mode was used at energy of 70eV.

2.9 GC-MS chemometric analysis

For each sample, a chromatogram and a series of mass spectra were generated. Peaks from the chromatograms were normalized with a sample; peaks from different samples were aligned to each other as a matrix. To ensure the peak identity, mass spectrum of each peak was analyzed by NIST98 search. The matrix was then analyzed by hierarchical cluster analysis with the use of square Euclidean distance and cosine of vectors of variables with between-groups linkage method in SPSS v13.0.

Chapter 3. Authentication of *Aconitum* by DNA Sequencing

3.1 Introduction

In this chapter, the use of DNA sequencing in authentication of Pharmacopoeia-listed *Aconitum* is discussed. Pharmacopoeia-listed *Aconitum*, namely *A. carmichaeli* and *A. kusnezoffii*, are often confused with other *Aconitum* in the market. Mis-identifying the species can cause intoxication (Section 1.3). Difficulty in identification is mainly due to the diversification of *Aconitum* species and non-distinct variations, and authentication is therefore required for the safe use of *Aconitum*.

Sequence of common markers, namely 5S spacer and *psb*A-*trn*H spacer, were obtained from different *Aconitum* samples. The ability of the markers in discriminating Pharmacopoeia-listed *Aconitum* from the unlisted ones was tested by sequence similarity and phylogram study.

3.2 Methods

Different plant materials, including medicinal materials and leaf specimen, were tested and the samples are listed in Section 2.1.1. DNA sequencing was performed as stated in Section 2.5. After sequencing, all the sequences from the same marker were aligned by ClustalW (Chenna *et al.*, 2003); and phylogram study was performed by MEGA 3.1 (Kumar *et al.*, 2004) using Kimura 2-parameter with neighbor-joining (NJ) and unweighted pair-group method using arithmetic mean (UPGMA). Phylogram study by Maximum Parsimony (MP) which is a discrete method was also performed by MEGA v3.1.

3.3 Results – 5S spacer

3.3.1 Sequence information

Sequence information was successfully obtained from 105 clones from 33 *Aconitum* samples, with an average of more than 3 clones per sample. The sequence ranged from 577bp to 600bp with an average of 628bp. The aligned length of 5S spacer was 725bp. The average G+C content was 46.9%. In the aligned sequences, the numbers of in-del sites, variable sites and informative sites were 342, 635 and 486

respectively. Table 4-9 shows a detailed comparison of sequence information of 5S spacer with other sequence markers. The aligned 5S spacer of *Aconitum* is shown in Appendix A.

3.3.2 Sequence similarity

The range of pairwise sequence similarities among different 5S spacer clones between *Aconitum* samples is shown in Table 3-1. The range of intra-sample similarities is shown also along the diagonal. For some samples, only a single clone was obtained and therefore no intra-sample similarity was calculated. A summary of the ranges of intra-specific and inter-specific similarities is shown in Table 3-2.

Table 3-1. Pairwise sequence similarities between 5S spacer from *Aconitum* samples. The range represents the values among different clones between two samples. Some intra-sample similarities are omitted due to the obtaining of only a single clone.

Sample	AC2	AC3	AC5	AC7	AC13	AC25	ACS	ACfz1	ACfz3	AK2	AK4	AK5	AK6c	AK7	AK8	AK9b	AK21
AC2	0.892-0.962	0.68-0.897	0.676-0.945	0.709-0.924	0.64-0.664	0.921-0.937	0.686-0.991	0.875-0.911	0.867-0.983	0.851-0.897	0.762-0.769	0.617-0.932	0.865-0.928	0.684-0.908	0.882-0.961	0.674-0.908	0.891-0.943
AC3		0.677-0.942	0.68-0.971	0.698-0.908	0.641-0.742	0.701-0.898	0.631-0.88	0.687-0.883	0.675-0.882	0.674-0.886	0.728-0.786	0.614-0.867	0.67-0.878	0.688-0.972	0.676-0.885	0.679-0.972	0.674-0.921
AC5			0.678-0.886	0.706-0.919	0.64-0.714	0.701-0.935	0.632-0.948	0.686-0.897	0.671-0.95	0.691-0.897	0.744-0.766	0.611-0.935	0.666-0.908	0.682-0.982	0.675-0.939	0.674-0.982	0.673-0.969
AC7				0.743-0.901	0.687-0.709	0.731-0.922	0.649-0.9	0.71-0.898	0.695-0.9	0.696-0.913	0.69-0.785	0.659-0.888	0.69-0.897	0.714-0.921	0.702-0.898	0.71-0.922	0.706-0.94
AC13					1	0.658	0.587-0.697	0.64-0.644	0.64-0.652	0.645-0.661	0.655	0.64-0.661	0.632-0.647	0.654-0.715	0.634-0.642	0.648-0.724	0.646-0.673
AC25						(- - -)	0.711-0.94	0.892-0.914	0.892-0.921	0.876-0.901	0.792	0.639-0.907	0.875-0.904	0.714-0.911	0.892-0.934	0.696-0.909	0.923-0.938
ACS							0.637-0.974	0.699-0.9	0.682-0.985	0.693-0.892	0.688-0.768	0.567-0.937	0.682-0.935	0.642-0.889	0.686-0.965	0.635-0.892	0.679-0.942
ACfz1								0.948	0.858-0.974	0.842-0.877	0.745-0.771	0.627-0.881	0.873-0.891	0.702-0.897	0.868-0.977	0.685-0.896	0.861-0.922
ACfz3									0.859-0.921	0.844-0.891	0.751-0.765	0.619-0.948	0.865-0.921	0.681-0.896	0.865-0.984	0.669-0.893	0.862-0.942
AK2										0.918	0.755-0.763	0.616-0.879	0.842-0.881	0.691-0.896	0.842-0.876	0.687-0.898	0.845-0.922
AK4											(- - -)	0.676-0.754	0.738-0.753	0.758-0.769	0.742-0.76	0.746-0.774	0.755-0.79
AK5												0.61-0.61	0.612-0.893	0.631-0.878	0.619-0.903	0.622-0.878	0.607-0.924
AK6c													0.855-0.912	0.679-0.883	0.857-0.947	0.664-0.884	0.882-0.91
AK7														0.711-0.982	0.681-0.897	0.691-0.983	0.678-0.932
AK8															0.874-0.914	0.671-0.897	0.87-0.929
AK9b																0.683-0.974	0.666-0.932
AK21																	0.897-0.991
AK23																	
AK24b																	
AY334493																	
AH11																	
AJ11																	
AN1																	
AN2																	
AV1																	
AV2																	
AV3																	
AV4																	
AKo1																	
AS2																	
AS3																	
AS4																	
AS11																	
AS12																	

Table 3-1. Pairwise sequence similarities between 5S spacer from *Aconitum* samples. The range represents the values among different clones between two samples. Some intra-sample similarities are omitted due to the obtaining of only a single clone.

(Continued)

Sample	AK23	AK24	AY334493	AH11	AH1	AN1	AN2	AV1	AV2	AV3	AV4	AKo1	AS2	AS3	AS4	ASf1	ASf2
AC2	0.672-0.696	0.85-0.91	0.874-0.884	0.678-0.903	0.857-0.908	0.876-0.91	0.888-0.91	0.884-0.909	0.884-0.911	0.692-0.883	0.881-0.905	0.693-0.736	0.697-0.923	0.696-0.9	0.674-0.895	0.875-0.932	0.894-0.918
AC3	0.685-0.781	0.673-0.951	0.677-0.87	0.674-0.971	0.67-0.947	0.688-0.974	0.702-0.948	0.693-0.972	0.694-0.95	0.681-0.926	0.695-0.948	0.657-0.713	0.662-0.882	0.698-0.944	0.682-0.942	0.667-0.918	0.697-0.9
AC5	0.67-0.752	0.675-0.958	0.678-0.881	0.68-0.975	0.678-0.959	0.697-0.983	0.706-0.961	0.703-0.985	0.702-0.963	0.687-0.934	0.701-0.958	0.686-0.716	0.658-0.918	0.697-0.952	0.676-0.948	0.664-0.923	0.698-0.911
AC7	0.711-0.795	0.696-0.923	0.703-0.889	0.716-0.914	0.695-0.916	0.717-0.919	0.726-0.919	0.725-0.919	0.72-0.919	0.711-0.894	0.722-0.916	0.689-0.726	0.684-0.896	0.725-0.912	0.718-0.907	0.692-0.937	0.725-0.918
AC13	0.67	0.646-0.665	0.634	0.657-0.673	0.628-0.661	0.657-0.667	0.664	0.658-0.664	0.658-0.666	0.652-0.714	0.67-0.671	0.628-0.639	0.621-0.759	0.669-0.721	0.667-0.687	0.632-0.673	0.66
AC25	0.693	0.876-0.91	0.876	0.698-0.904	0.88-0.906	0.902-0.91	0.908	0.906-0.914	0.908-0.913	0.706-0.888	0.902-0.903	0.706-0.718	0.718-0.902	0.721-0.899	0.696-0.894	0.902-0.924	0.914
ACS	0.616-0.753	0.68-0.891	0.696-0.877	0.623-0.884	0.689-0.891	0.705-0.891	0.716-0.892	0.713-0.894	0.716-0.894	0.652-0.87	0.707-0.886	0.642-0.707	0.647-0.924	0.648-0.885	0.636-0.876	0.681-0.928	0.716-0.897
ACTz1	0.68-0.686	0.855-0.899	0.858-0.863	0.685-0.889	0.856-0.897	0.883-0.897	0.891-0.895	0.892-0.9	0.888-0.9	0.697-0.876	0.888-0.894	0.69-0.705	0.704-0.974	0.701-0.889	0.689-0.884	0.852-0.979	0.891-0.897
ACTz3	0.671-0.689	0.847-0.895	0.863-0.878	0.67-0.893	0.853-0.894	0.877-0.893	0.886-0.893	0.88-0.894	0.882-0.896	0.691-0.874	0.878-0.89	0.684-0.713	0.696-0.98	0.692-0.891	0.667-0.88	0.854-0.987	0.889-0.897
AK2	0.684-0.699	0.847-0.897	0.848-0.862	0.673-0.895	0.843-0.894	0.867-0.897	0.877-0.895	0.87-0.9	0.872-0.897	0.693-0.87	0.87-0.892	0.701-0.72	0.692-0.873	0.704-0.886	0.689-0.884	0.842-0.914	0.877-0.898
AK4	0.682	0.734-0.765	0.751	0.743-0.764	0.736-0.765	0.757-0.763	0.768	0.763-0.766	0.765-0.773	0.745	0.76-0.766	0.676-0.68	0.738-0.755	0.76-0.798	0.737-0.76	0.742-0.776	0.768
AK5	0.665-0.696	0.61-0.878	0.632-0.878	0.628-0.875	0.61-0.878	0.626-0.88	0.64-0.881	0.634-0.883	0.635-0.881	0.616-0.854	0.63-0.873	0.618-0.699	0.595-0.944	0.637-0.869	0.629-0.864	0.597-0.899	0.633-0.886
AK6c	0.658-0.674	0.838-0.881	0.866-0.877	0.67-0.887	0.839-0.878	0.866-0.881	0.868-0.88	0.87-0.884	0.866-0.881	0.685-0.855	0.863-0.875	0.684-0.697	0.688-0.895	0.695-0.873	0.669-0.866	0.846-0.905	0.876-0.888
AK7	0.698-0.765	0.69-0.963	0.701-0.879	0.692-0.975	0.69-0.959	0.714-0.985	0.72-0.961	0.717-0.983	0.717-0.961	0.7-0.934	0.717-0.959	0.683-0.719	0.665-0.896	0.717-0.955	0.695-0.95	0.681-0.922	0.715-0.91
AK8	0.663-0.691	0.835-0.896	0.863-0.874	0.677-0.893	0.841-0.896	0.864-0.896	0.869-0.894	0.872-0.897	0.867-0.897	0.686-0.873	0.863-0.892	0.688-0.706	0.69-0.979	0.699-0.893	0.673-0.882	0.858-0.984	0.886-0.896
AK9b	0.688-0.771	0.671-0.959	0.676-0.877	0.682-0.982	0.669-0.958	0.689-0.982	0.7-0.959	0.697-0.985	0.694-0.961	0.68-0.931	0.694-0.958	0.68-0.714	0.653-0.892	0.699-0.953	0.682-0.947	0.67-0.922	0.694-0.908
AK21	0.67-0.708	0.847-0.932	0.869-0.923	0.671-0.932	0.856-0.932	0.876-0.937	0.88-0.934	0.875-0.938	0.875-0.935	0.69-0.907	0.874-0.931	0.688-0.736	0.695-0.923	0.693-0.924	0.668-0.922	0.869-0.975	0.891-0.964
AK23	(- - -)	0.672-0.706	0.677	0.695-0.743	0.677-0.705	0.701-0.705	0.704	0.704	0.704-0.705	0.687-0.744	0.702-0.706	0.699-0.709	0.648-0.747	0.705-0.741	0.698-0.744	0.658-0.704	0.689
AK24b		0.931-0.99	0.843-0.879	0.667-0.956	0.9-0.963	0.926-0.966	0.932-0.964	0.928-0.956	0.929-0.966	0.677-0.956	0.936-0.977	0.691-0.722	0.69-0.894	0.703-0.972	0.68-0.966	0.834-0.927	0.878-0.913
AY334493			(- - -)	0.68-0.876	0.846-0.879	0.878-0.885	0.881	0.878-0.884	0.879-0.882	0.691-0.854	0.876	0.696-0.701	0.694-0.864	0.709-0.872	0.679-0.868	0.866-0.908	0.898
AH11				0.688-0.98	0.67-0.955	0.687-0.976	0.702-0.956	0.698-0.98	0.7-0.958	0.679-0.928	0.696-0.95	0.675-0.713	0.656-0.889	0.7-0.95	0.69-0.94	0.665-0.922	0.696-0.908
AH1					0.921-0.996	0.919-0.961	0.929-0.982	0.924-0.963	0.926-0.988	0.676-0.942	0.921-0.964	0.691-0.717	0.693-0.894	0.705-0.961	0.676-0.955	0.835-0.921	0.878-0.908
AN1						0.98-0.988	0.952-0.963	0.972-0.982	0.948-0.964	0.703-0.94	0.95-0.966	0.706-0.719	0.709-0.893	0.716-0.96	0.69-0.956	0.858-0.927	0.903-0.916
AN2							(- - -)	0.959-0.963	0.977-0.982	0.707-0.939	0.96-0.964	0.717-0.722	0.723-0.892	0.73-0.961	0.706-0.955	0.87-0.923	0.91
AV1								0.98	0.958-0.961	0.707-0.931	0.952-0.956	0.712-0.72	0.715-0.897	0.722-0.955	0.697-0.947	0.869-0.927	0.908-0.916
AV2									0.979-0.983	0.706-0.944	0.956-0.971	0.708-0.719	0.715-0.899	0.723-0.966	0.7-0.958	0.867-0.924	0.908-0.911
AV3										0.689	0.71-0.956	0.696-0.715	0.673-0.871	0.709-0.953	0.688-0.944	0.686-0.899	0.709-0.889
AV4											0.984	0.711-0.721	0.714-0.89	0.724-0.977	0.7-0.98	0.861-0.921	0.907-0.908
AKo1												0.907-0.953	0.681-0.702	0.685-0.717	0.682-0.713	0.687-0.727	0.71-0.719
AS2													0.684-0.872	0.679-0.891	0.652-0.88	0.677-0.99	0.711-0.899
AS3														0.722	0.705-0.963	0.684-0.917	0.719-0.907
AS4															0.7	0.661-0.911	0.693-0.898
ASf1																0.86-0.912	0.877-0.958
ASf2																	(- - -)

Table 3-2. The intra-specific and inter-specific pairwise similarities between clones of 5S spacer from *Aconitum* species

Species	Intra-specific pairwise similarity	Pairwise similarity with other species
A. carmichaeli	0.637-0.974	0.623-0.942
A. kusnezoffii	0.845-0.991	0.671-0.942
A. hemsleyanum	0.670-0.996	0.671-0.988
A. nagarum	0.952-0.988	0.687-0.982
A. vilmorinianum	0.689-0.984	0.652-0.988
A. coreanum	0.907-0.953	0.642-0.736

3.3.3 Phylogram study

Phylograms of 5S spacer of *Aconitum* were constructed by using three methods with bootstrap tested by 1000 replicates: neighbor-joining (NJ), unweighted uair-group method using arithmetic mean (UPGMA) and maximum parsimony (MP) are shown in Figure 3-1, Figure 3-2 and Figure 3-3 respectively. A sequence from Genbank with accession number AY334493 was also used for phylogram analysis.

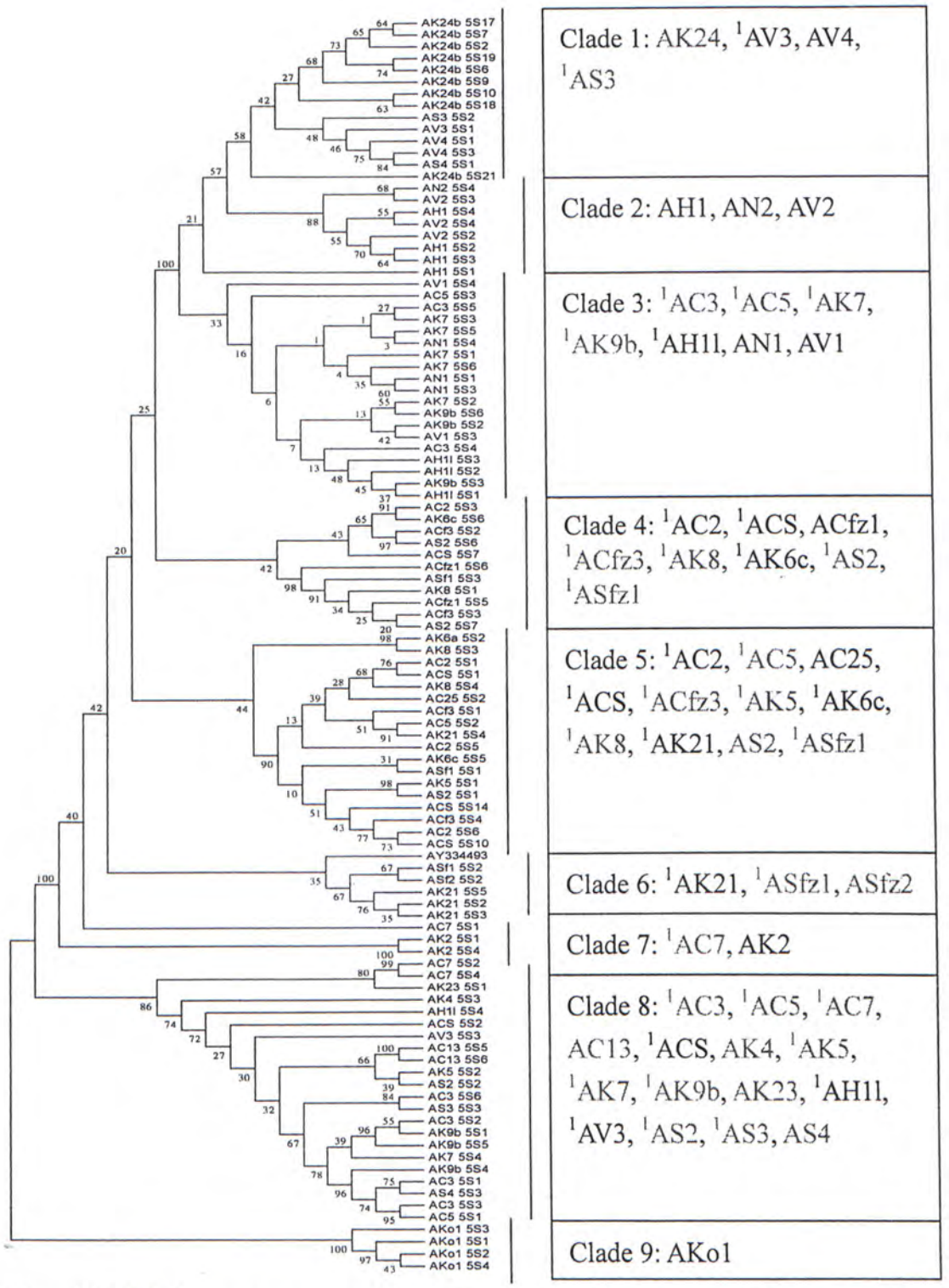

Figure 3-1. Phylogram constructed from *Aconitum* nuclear ribosomal 5S spacer using neighbor-joining method with bootstrap tested by 1000 replicates. Only the tree topology is shown here for illustration. Each taxon represents an individual clone from each sample. Sequences from Genbank are included. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents clones of a sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

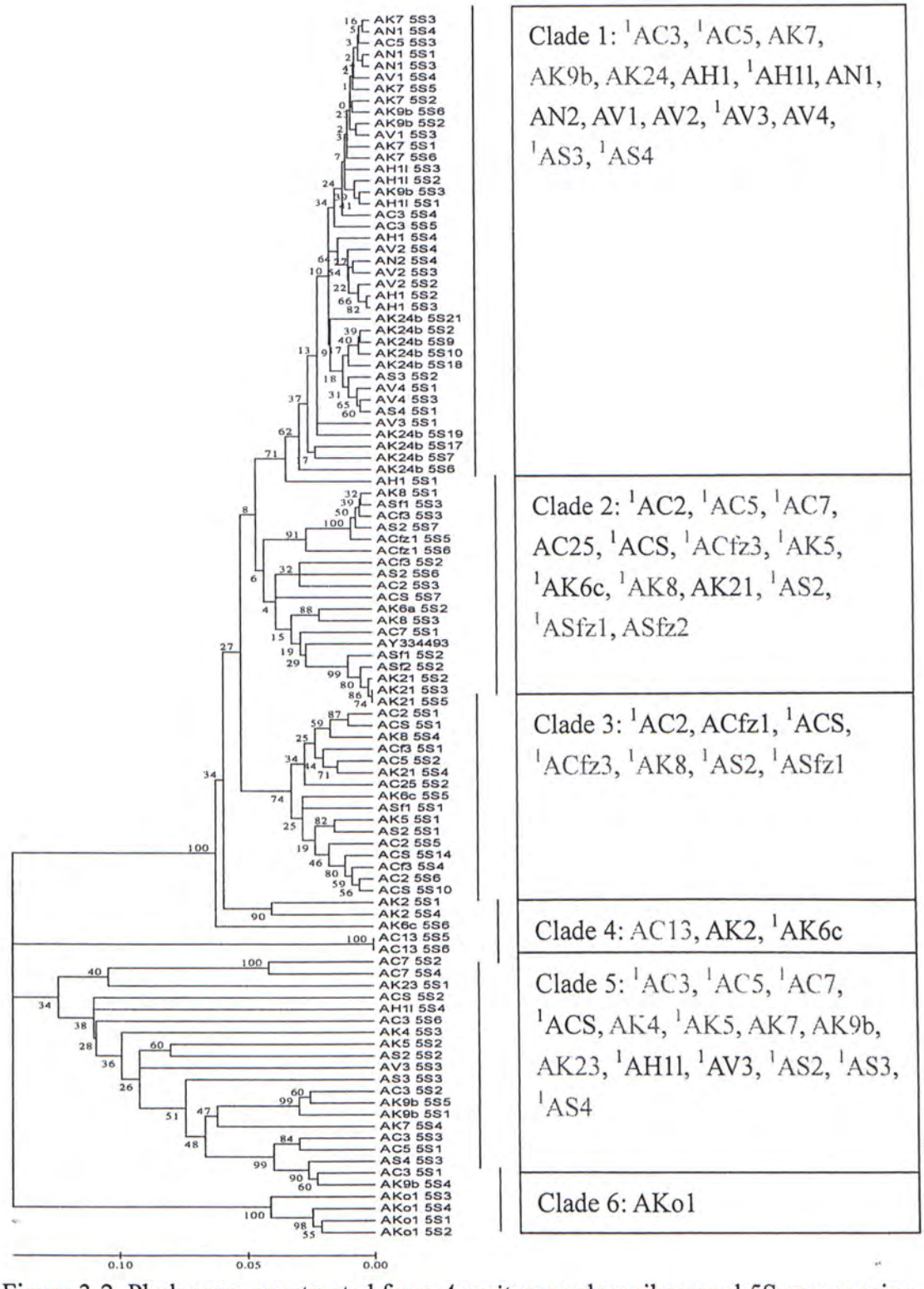

Figure 3-2. Phylogram constructed from *Aconitum* nuclear ribosomal 5S spacer using UPGMA method with bootstrap tested by 1000 replicates. Each taxon represents an individual clone from each sample. Sequences from Genbank are included. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents clones of a sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

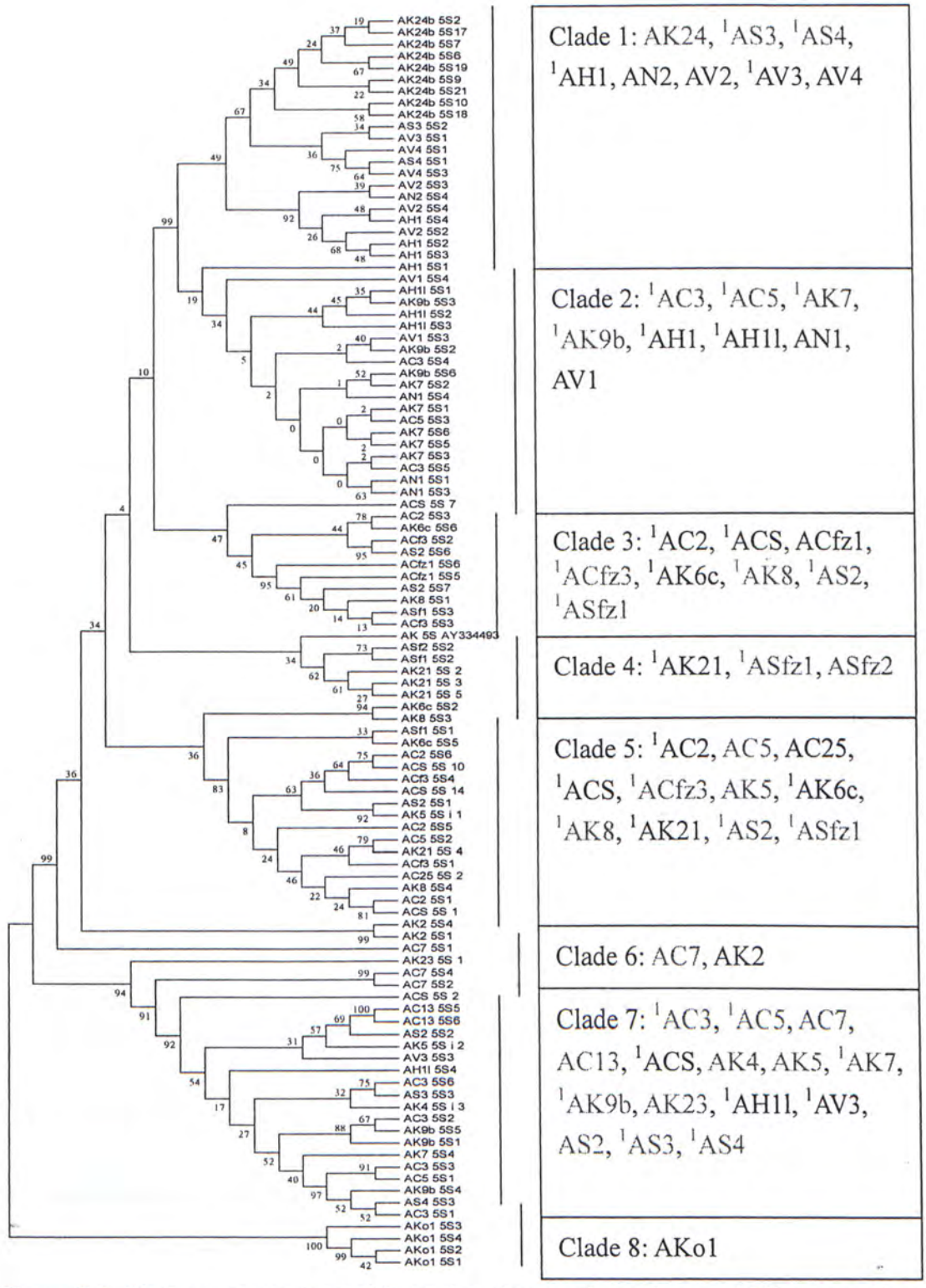

Figure 3-3. Phylogram constructed from *Aconitum* nuclear ribosomal 5S spacer using maximum parsimony method with bootstrap tested by 1000 replicates. Each taxon represents an individual clone from each sample. Sequences from Genbank are included. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents clones of a sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

3.4 Results – *psb*A-*trn*H

3.4.1 Sequence information

Sequence information was successfully obtained from 41 *Aconitum* samples. The sequence lengths ranged from 222bp to 239bp with an average of 230bp. The aligned length of *psb*A-*trn*H spacer was 264bp. The average G+C content was 31.5%. In the aligned sequences, the number of in-del sites, variable sites and informative sites were 53, 58 and 26 respectively. Table 4-9 shows a detailed comparison of sequence information of *psb*A-*trn*H spacer with other sequence markers. The aligned *psb*A-*trn*H spacer of *Aconitum* is shown in Appendix B.

3.4.2 Sequence similarity

The pairwise sequence similarities between *psb*A-*trn*H from *Aconitum* samples are shown in Table 3-3. A summary of the ranges of intra-specific and inter-specific similarities is shown in Table 3-4.

Table 3-3. Pairwise sequence similarities between *psb*A-*trn*H from *Aconitum* samples

Table 3-4. The intra-specific and inter-specific pairwise similarities between *psb*A-*trn*H spacer from *Aconitum* species

Species	Intra-specific pairwise similarity	Pairwise similarity with other species
A. carmichaeli	0.930-1	0.794-1
A. kusnezoffii	0.909-0.995	0.791-1
A. hemsleyanum	---	0.841-1
A. nagarum	0.995	0.836-1
A. vilmorinianum	0.991	0.841
A. coreanum	---	0.841-0.986

3.4.3 Phylogram study

Phylograms of *psb*A-*trn*H spacer of *Aconitum* were constructed by using three methods: neighbor-joining (NJ), unweighted pair-group method using arithmetic mean (UPGMA) and maximum parsimony (MP) are shown in Figure 3-4, Figure 3-5 and Figure 3-6 respectively. Sequences from Genbank with accession number AF216559, AF216563, AF216567, AF216573, AF216575, AF216576, AF216577 and AF216578 were also used for phylogram analysis.

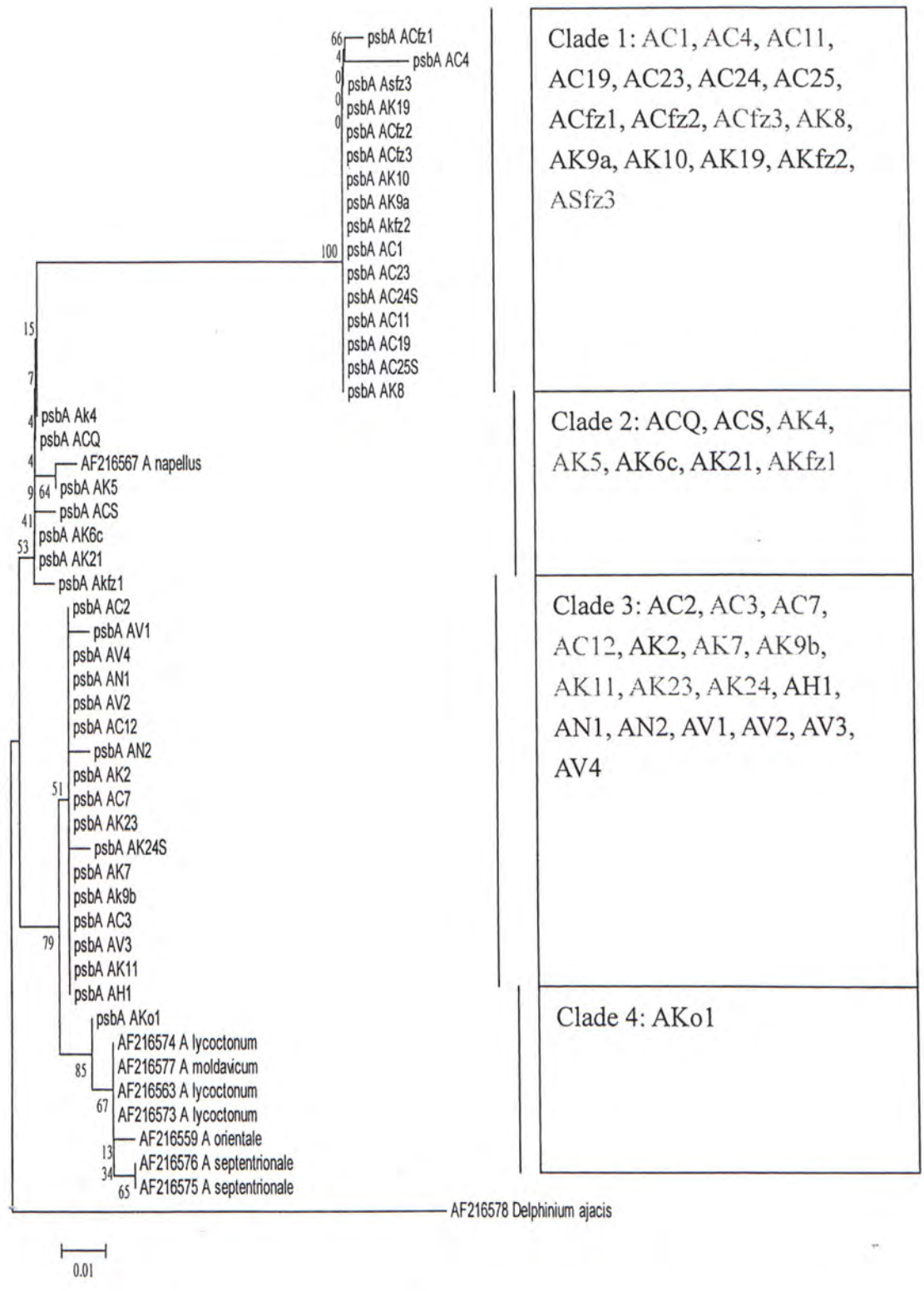

Figure 3-4. Phylogram constructed from *Aconitum* chloroplast *psb*A-*trn*H spacer using neighbor-joining method. Sequences from Genbank are included. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. Samples with unsure identities are shaded in grey.

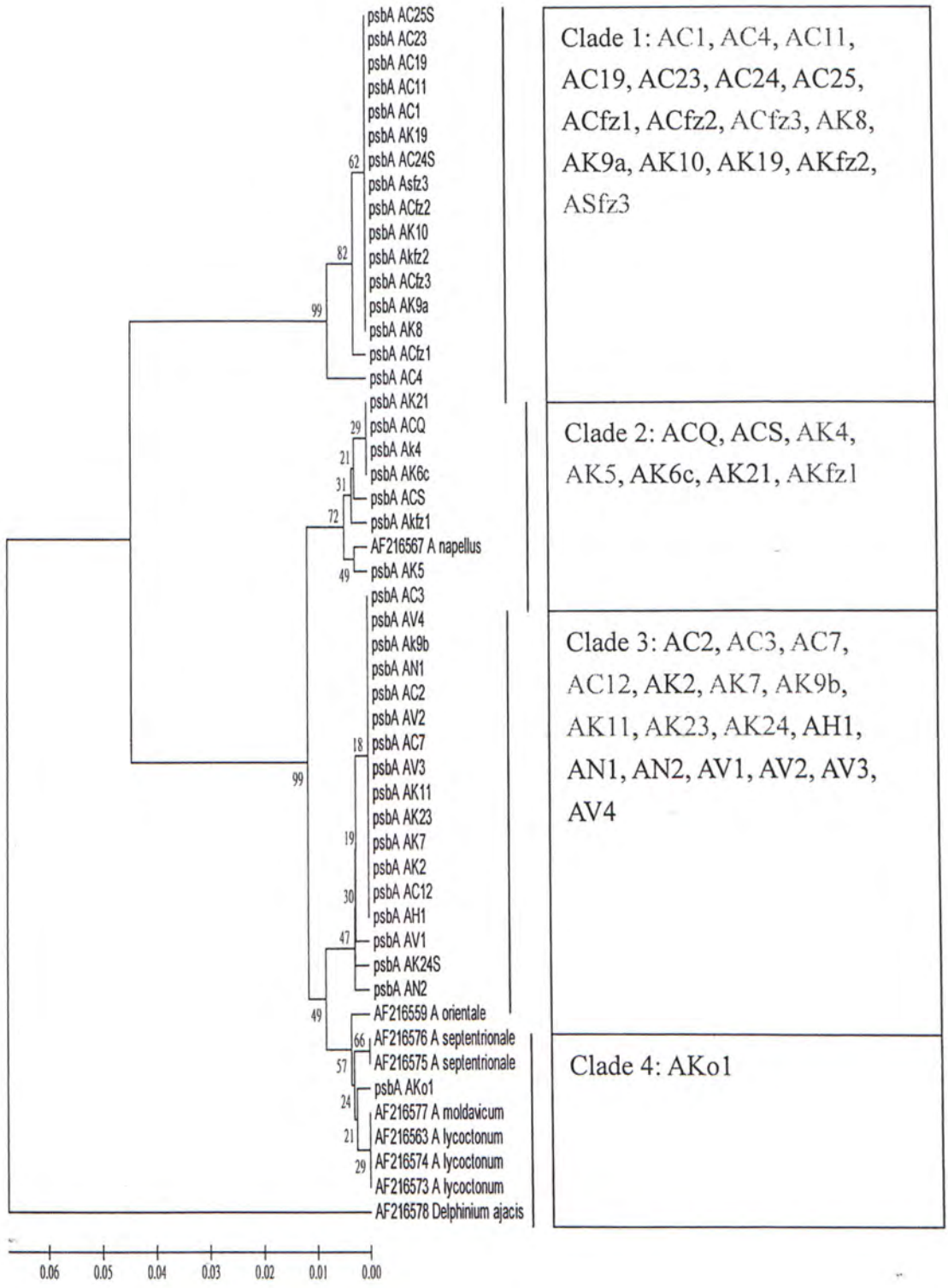

Figure 3-5. Phylogram constructed from *Aconitum* chloroplast *psb*A-*trn*H spacer using UPGMA method with bootstrap tested by 1000 replicates. Sequences from Genbank are included. Sample codes listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. Samples with unsure identities are shaded in grey.

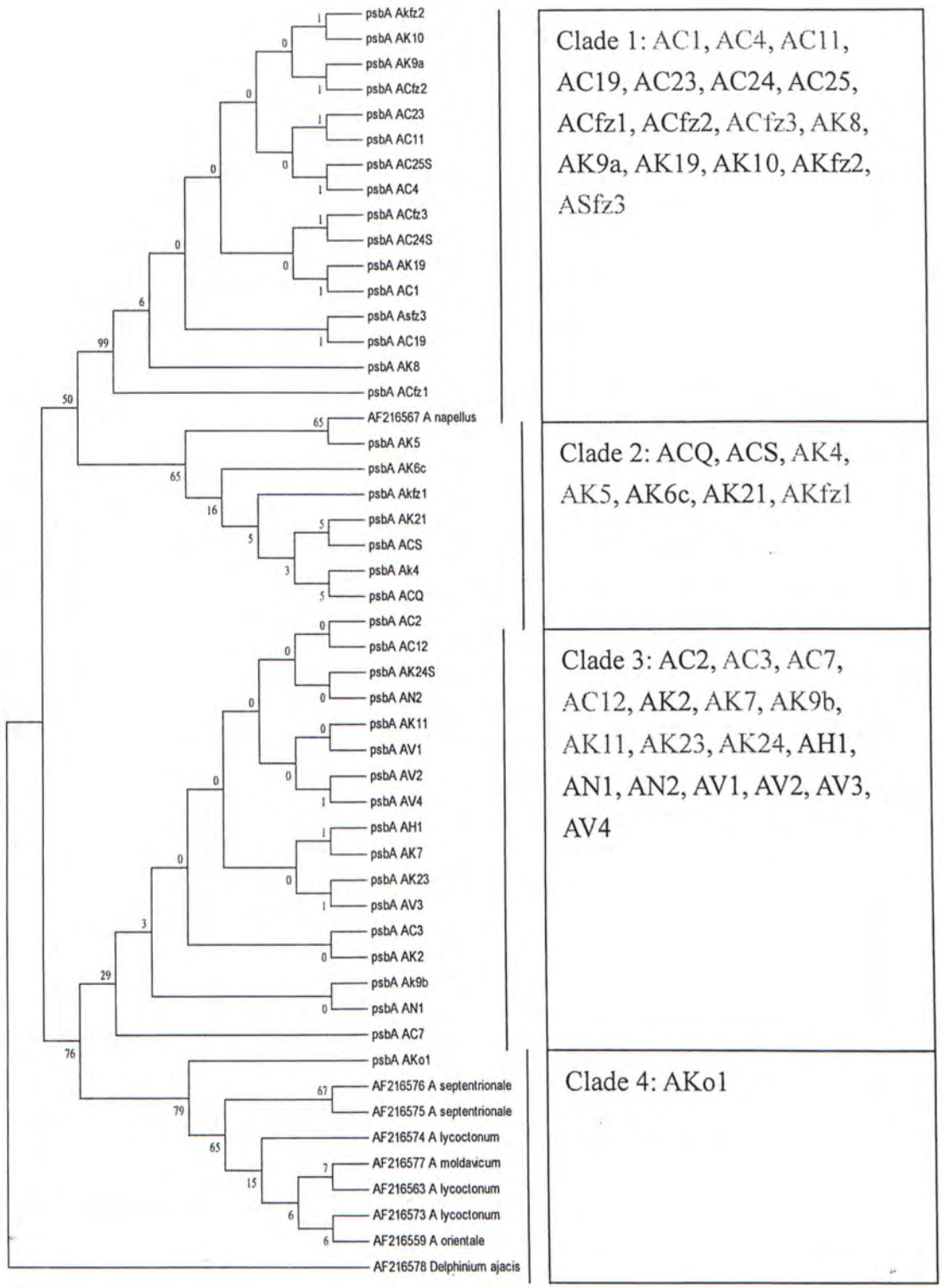

Figure 3-6. Phylogram constructed from *Aconitum* chloroplast *psb*A-*trn*H spacer using maximum parsimony method with bootstrap tested by 1000 replicates. Sequences from Genbank are included. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. Samples with unsure identities are shaded in grey.

3.5 Discussion

3.5.1 Overview of nuclear ribosomal 5S spacer

5S spacer was a highly variable marker across genus *Aconitum* with 87.6% substitution sites and 47.2% in-del sites. The intra-individual variation of 5S spacer was also high. The intra-individual similarity could be as low as 70% in many of the samples studied. In the phylogram studies, over half of the samples had clones in more than one clade, meaning that not only the variation among copies within a sample was high, but the number of multiple copies was also high.

3.5.2 Extensive polymorphism of 5S spacer

Extensive polymorphism in nuclear ribosomal DNA within individuals of *Aconitum* was reported as a result of non-concerted evolution of 5S spacer, inter-specific hybridization or evolution of pseudogenes (Kita and Ito, 2000). That is in line with a general hypothesis that *Aconitum* species have undergone various hybridization, resulting in non-discrete morphological variations. Some *Aconitum* species are also used in gardening which may also have undergone artificial hybridization before being used for medicinal purpose. Pseudogene is another problem of using 5S spacer for authentication. 5S rDNA are organized in tandem repeats throughout the

genome. Different loci can have different forms of 5S rDNA. However, not all loci can produce mature 5S rRNA, and in different stages and organs, different 5S transcripts are produced (Cloix *et al.*, 2001). Those pseudogenes of 5S spacer may have evolved extensively, causing high copy numbers and a high degree of variation among clones of 5S spacer within *Aconitum*.

Some 5S spacer clones formed polyphyletic clades within the whole phylograms. In NJ Clade 4 and 5 (Figure 3-1), UPGMA Clade 2 and 3 (Figure 3-2), as well as MP Clade 3 and 5 (Figure 3-3), clones from some samples, like AC2, ACfz3, AK6c, AK8 and ASfz1, always clustered together in the two clades. That implied a recent evolved 5S spacer and also interspecific hybridization forming polyploids.

Heterogeneity and/or pseudogenes of 5S spacer were suspected to form NJ Clade 8 (Figure 3-1), UPGMA Clade 5 (Figure 3-2) and MP Clade 7 (Figure 3-3), because the clades contained clones from samples which also occurred in other clades. As revealed by the phylograms, these suspected clades of psuedogenes branched out near the root, and this implied the formation of these pseudogenes could be earlier than the occurance of hybridization.

3.5.3 Distribution of samples in the phylograms constructed by 5S spacer

The distribution of samples in the three phylograms was similar. The most distinctive feature was the isolation of clade of *A. coreanum* (AKo1). In the three phylograms, it formed only one clade on its own. Unlike other *Aconitum* samples, its four clones did not show a large variation.

Other non-Pharmacopoeia-listed species *A. hemsleyanum*, *A. nagarum* and *A. vilmorinianum* formed Clade 1 in UPGMA (Figure 3-2) or sister clades in NJ Clade1, 2 and 3 (Figure 3-1) and MP Clade 1 and 2 (Figure 3-3). It showed that they were closely linked.

The Pharmacopoeia-listed species *A. carmichaeli* and *A. kusnezoffii* formed polyphyletic clades in the three trees: NJ Clade 4, 5 and 6 (Figure 3-1), UPGMA Clade 2 and 3 (Figure 3-2) and MP Clade 3, 4 and 5 (Figure 3-3). However, there was no simple marker which can distinguish *A. carmichaeli* and *A. kusnezoffii* from one another and therefore authentication of Pharmacopoeia-listed *Aconitum* species requires other markers.

3.5.4 Utility of 5S spacer for authentication

The utility of 5S spacer for authentication is greatly hindered by the extensive polymorphism and psuedogenes. Authentication of *Aconitum* using 5S spacer requires cloning in which functional clones are hopefully obtained. Direct sequencing of 5S spacer cannot lead to species information. Although 5S spacer can provide supporting evidence to show whether the sample is Pharmacopoeia-listed species or not, it still cannot distinguish *A. carmichaeli* and *A. kusnezoffii* from one another.

3.5.5 Overview of *psb*A-*trn*H spacer

*psb*A-*trn*H spacer from chloroplast genome is another well-studied marker in this project. Markers from chloroplast genome were thought to be less variable and thus, less useful in inferring phylogenetic relationship at a low taxonomic level (Species-Genus). But a recent study reported that *psb*A-*trn*H spacer was still variable enough for revealing relationships at a species level for some plant species (Utelli *et al.*, 2000).

Unlike 5S spacer, *psb*A-*trn*H spacer did not have the problem of multiple copies or extensive polymorphisms in *Aconitum*.

3.5.6 Distribution of samples in the phylograms constructed by *psb*A-*trn*H spacer

Samples were grouped exactly in the same way in the three phylograms constructed by NJ (Figure 3-4), UPGMA (Figure 3-5) and MP (Figure 3-6).

Clade 4 of the three phylograms (Figure 3-4, Figure 3-5 and Figure 3-6) consisted of *A. coreanum* and sequences from another subgenus *Lycoctonum*. Together with the analysis from 5S spacer, *A. coreanum* can be well distinguished from other *Aconitum* species. Clade 3 (Figure 3-4, Figure 3-5 and Figure 3-6), which consisted of *A. hemsleyanum*, *A. nagarum* and *A. vilmorinianum*, was sister of Clade 4. Clustered with non-Pharmacopoeia-listed species in *psb*A-*trn*H, samples AC3, AK7, AK9b and AK24 also had the same pattern in 5S spacer.

Clade 1 and Clade 2 (Figure 3-4, Figure 3-5 and Figure 3-6), which contained *A. carmichaeli* and *A. kusnezoffii* samples only, were sister clades. *A. napellus* which is a European species was also used in phylogram study. It was clustered within Clade 2 but it was still sister clade of a sub-clade of *A. carmichaeli* and *A. kusnezoffii* with a fairly high bootstrap value support. This implied a relatively close relationship of *A. carmichaeli* and *A. kusnezoffii* (Ser. *Inflata*) with *A. napellus*.

3.5.7 A distinctive region of inversion

Interestingly, although Clade 1 and Clade 2 were sister clades, they were supported by fairly low values of bootstrap tests, and this situation was not seen in 5S spacer. The two clades were distinguished by a distinctive 25-bp inversion at position 74 in the alignment (Figure 3-7).

Type1	CCCAGCCTC--TTAACAGAACAAGAAATTG
Type2	CCCTATTTC--TTGTTCTGTTAAGAGGCTG

Figure 3-7. A distinctive region of 25-bp inversion in *psb*A-*trn*H position 74 in *Aconitum*

If only this 25-bp region was considered, all samples could be divided into two groups: one (Type 1 in Figure 3-7) with *A. carmichaeli* and *A. kusnezoffii* samples and another (Type 2 in Figure 3-7) with *A. carmichaeli*, *A. kusnezoffii* and other samples including those in Subgen. *Lycoctonum*. Geographic distribution is unlikely possible to explain this as it has no direct relation with this 25-bp inversion. The inversion also suggests that it was not resulted from random mutations in the spacer, but instead the inversion should be a single genetic event during the evolution of *Aconitum*. This kind of minute inversion was suggested to be a commonly found feature which occurs in non-coding DNA in chloroplast and was probably due to hairpin secondary structures (Kelchner and Wendel, 1996). This inversion occurred

only in some but not other samples of *A. carmichaeli* and *A. kusnezoffii*. This suggested that the two species might not have a monophyletic origin. However, more evidence is required to confirm their origin which may involve a more detailed study of their genomes.

3.5.8 Utility of *psb*A-*trn*H for authentication

The consistency in copies of *psb*A-*trn*H spacer allows direct sequencing of PCR products, in contrast to the polymorphism in 5S spacer which requires cloning procedures. This advantage of *psb*A-*trn*H spacer enables fast authentication and easy data interpretation. *psb*A-*trn*H spacer is able to identify non-Pharmacopoeia-listed species of *Aconitum*. However, it still cannot distinguish *A. carmichaeli* and *A. kusnezoffii* from one another. Owing to the difficulty in discriminating of the two closely related species by readily-available markers, novel markers have to be screened for authentication of the two medicinal species.

Chapter 4. Screening for Novel Markers for Authentication of *Aconitum*

4.1 Introduction

Although, as mentioned in Chapter 3, 5S spacer and *psb*A-*trn*H spacer could roughly identify non-Pharmacopeia-listed species from the listed ones, the discrimination between *A. carmichaeli* and *A. kusnezoffii* was not yet achieved. It was therefore necessary to screen for novel markers for the authentication of *Aconitum*.

In this chapter, a technique which allows the screening of differential sequences was applied. This technique made use of subtractive hybridization to achieve the screening. The mechanism is presented in Section 1.15.2.

After preliminary screening, useful markers were further selected. Primers were designed and used in sequencing the respective regions among the *Aconitum* samples and the usefulness of each marker was confirmed by phylogram analysis.

4.2 Methods

Sample *A. carmichaeli* ACfz2 and *A. kusnezoffii* AK2 were selected as the tester and driver respectively. Their DNA was extracted by the method described in Section 2.2. The two genomes were then subtracted as stated in Section 2.6 and a subtraction library was constructed. The screening for differential markers was performed as Section 2.7. Primers designed for several selected regions are shown in Table 4-2. The regions among the *Aconitum* samples (Section 2.1.1) were sequenced as described in Section 2.5.

After obtaining the sequences, those from the same markers were aligned by ClustalW (Chenna *et al.*, 2003); and phylogram study was performed by MEGA 3.1 (Kumar *et al.*, 2004).

4.3 Results – subtracted clones

A library consists of subtracted clones between ACfz2 and AK2 was established and 32 clones were sequenced, analyzed and matched with BLASTN provided by National Center for Biotechnology Information (NCBI). Sequence length,

percentage G+C content and BLASTN results are shown in Table 4-1. Based on the results, primers were designed for amplification of the six selected regions. The primer sequences are listed in Table 4-2. The sequences of the subtracted clones are shown in Appendix C.

The feasibility of certain regions to be used as markers was further tested. The primers were used to amplify against the DNA extracts of both ACfz2 and AK2. Six pairs of primers were tested and the results are shown in Figure 4-1. If the primers could give PCR products, those products from ACfz2 and AK2 would be sequenced and compared. Only those regions which contained a significant amount of polymorphism would be chosen for final screening. SSH6, SSH15 and SSH45 were selected.

The selected primers were used to sequence the subtracted regions from other *Aconitum* samples. The feasibility of those regions to be used as markers was examined by sequence similarity and phylogram study.

Table 4-1. Information of the sequenced subtracted clones of *Aconitum*. Blastn results are generated in April, 2006.

Subtracted clones	Total	G+C%	Blastn result
SSH1	131	48.9	BT009460 *Triticum aestivum* clone wpi1s.pk008.k5:fis
SSH2	564	30	AC157894 *Medicago truncatula* chromosome 7 BAC clone mth2-85d15
SSH3	274	44.2	AY631962 *Aconitum napellus* clone 25 microsatellite sequence
SSH4	190	44.2	AC098833 *Oryza sativa* (japonica cultivar-group) chromosome 5 clone OJ1288_A07
SSH5	235	40.8	AY631959 *Aconitum napellus* clone 7 microsatellite sequence
SSH6	281	39.5	AY757824 *Acorus gramineus* Ycf3 (ycf3) gene
SSH7	128	18.8	X13159 Maize chloroplast genes ndhD, ndhE and psaC
SSH8	306	31	AJ428413 *Calycanthus fertilis* var. *ferax* complete chloroplast genome
SSH9	283	40	AY757824 *Acorus gramineus* Ycf3 (ycf3) gene
SSH10	339	38.9	AY237140 *Nelumbo lutea* ribosomal protein S12 (rps12) gene, partial cds; ribosomal protein S7 (rps7) gene, complete cds; and NADH dehydrogenase subunit B (ndhB) gene, partial cds
SSH13	175	40	AC141111 *Medicago truncatula* clone mth2-20c17
SSH15	489	34.3	AJ131449 *Crocus vernus* dispersed repetitive DNA sequence, pCvKB5

Table 4-1. Information of the sequenced subtracted clones of *Aconitum*. Blastn results are generated in April, 2006.

Subtracted clones	Total	G+C%	Blastn result
(Continued)			
SSH16	180	43.9	DQ013044 *Lactuca sativa* NADH dehydrogenase subunit 2 (ndhB) gene, partial cds; ribosomal protein S7 (rps7) gene, complete cds; ribosomal protein S12 (rps12) gene, partial cds; tRNA-Val, 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; 23S ribosomal RNA gene, partial sequence; and unknown gene; chloroplast
SSH17	144	44.5	No Match
SSH18	279	40.1	Y18934 *Solanum nigrum* chloroplast tRNA-Ala, tRNA-Ile, 16S rRNA, tRNA-Val, rps12, rps7, ndhB genes
SSH25	60	55	No Match
SSH27	259	35.5	No Match
SSH34	113	44.2	No Match
SSH35	202	44	AP006444 *Brassica napus* mitochondrial DNA
SSH36	156	43.6	No Match
SSH37	324	43.5	DQ069574 *Ranunculus macranthus* cytochrome b6 (petB) gene
SSH38	191	44	X68256 *Brassica campestris* mitochondrion gene for NADH dehydrogenase subunit 6
SSH39	197	35.5	AJ131449 *Crocus vernus* dispersed repetitive DNA sequence, pCvKB5
SSH40	150	36.7	AY237132 *Berberis aquifolium* ribosomal protein S12 (rps12) gene, partial cds; ribosomal protein S7 (rps7) gene, complete cds; and NADH dehydrogenase subunit B (ndhB) gene, partial cds; chloroplast genes encoding chloroplast proteins

Table 4-1. Information of the sequenced subtracted clones of *Aconitum*. Blastn results are generated in April, 2006.

Subtracted clones	Total	G+C%	Blastn result
(Continued)			
SSH41	358	31.8	AY780259 *Eucalyptus globulus* subsp. globulus chloroplast, complete genome
SSH42	299	45.5	DQ069544 *Ranunculus macranthus* RNA polymerase beta" chain (rpoC2) gene partial cds; chloroplast.
SSH44	236	45.7	NM_128279 *Arabidopsis thaliana* hydrolase/ protein serine/threonine phosphatase AT2G27210 mRNA, complete cds.
SSH45	410	36.4	AJ131449 *Crocus vernus* dispersed repetitive DNA sequence, pCvKB5.
SSH46	177	41.8	No Match
SSH49	504	43	No Match
SSH51	306	33.7	DQ384529 *Panax ginseng* clone PG6L-6 unknown mRNA
SSH54	348	37.6	DQ424856 *Vitis vinifera* cultivar Maxxa chloroplast, complete genome

Table 4-2. Primer sequences which were designed for PCR of the subtracted regions

Region	Primer Name	Sequence
SSH2	SSH-A-2F	ACC AAT GGT TTA TCC AAA T
	SSH-A-2R	ACT AAC CCC AAA CAT ATC T
SSH6	SSH-A-6F	TTT GAT ACC CGT ATA ACC A
	SSH-A-6R	ACA TGA GAT TTT CAC CTC A
SSH15	SSH-A-15F	AGA GAC ATC ATC AAT CCC G
	SSH-A-15R	TGT ATG CAT ATA TCC AAC C
SSH18	SSH-A-18F	CCC GGG ACA GAG TCT ATA CAA
	SSH-A-18R	ATG AGA GAA GCA AGG AGG TC
SSH27	SSH-A-27F	TTG TAA GTC AAT CCC ATC A
	SSH-A-27R	ATT TTC CAT TAA ATT TCC A
SSH45	SSH-A-45F	CAG TTG TAG TGT GAG TCG G
	SSH-A-45R	TCA TCC TTT TTG TCA TGT C

Figure 4-1. Gel photo showing the PCR results using designed SSH primers on *A. carmichaeli* ACfz2 and *A. kusnezoffii* AK2. Lane 1: SSH2 primers on ACfz2. Lane 2: SSH2 primers on AK2. Lane 3: SSH6 primers on ACfz2. Lane 4: SSH6 primers on AK2. Lane 5: SSH15 primers on ACfz2. Lane 6: SSH15 primers on AK2. Lane M: 100bp ladder (The brightest band represents 700bp). Lane 7: SSH18 primers on ACfz2. Lane 8: SSH18 primers on AK2. Lane 9: SSH27 primers on ACfz2. Lane 10: SSH27 primers on AK2. Lane 11: SSH45 primers on ACfz2. Lane 12: SSH45 primers on AK2.

4.4 Results – SSH6

4.4.1 Sequence information

Sequence information was successfully obtained from 12 *Aconitum* samples. The sequence lengths ranged from 213bp to 216bp with an average of 215bp. The aligned length of SSH6 was 218bp. The average G+C content was 36.8%. In the aligned sequences, the number of in-del sites, variable sites and informative sites were 9, 12 and 2 respectively. Table 4-9 shows a detailed comparison of sequence

information of SSH6 with other sequence markers. The aligned SSH6 of *Aconitum* is shown in Appendix D.

Phylogram study was not carried out for SSH6 since the number of informative sites was as few as 2 (2.2%).

4.4.2 Sequence similarity

The pairwise sequence similarities of the different *Aconitum* samples are shown in Table 4-3. A summary of the ranges of intra-specific and inter-specific similarities is shown in Table 4-4.

Table 4-3. Pairwise sequence similarities between SSH6 regions from *Aconitum* samples

Sample	SSH6_AC19_1	SSH6_AC19_2	SSH6_ACfz2_1	SSH6_ACfz2_2	SSH6_ACQ_1	SSH6_ACQ_2	SSH6_ACS_1	SSH6_ACS_2	SSH6_AK2_1	SSH6_AK2_2	SSH6_AK9a_1	SSH6_AK9a_2	SSH6_AH1	SSH6_AK24_1	SSH6_AK24_2	SSH6_AKo1	SSH6_AN1	SSH6_AV1
SSH6_AV2	0.98	0.98	0.97	0.98	0.98	0.98	0.98	0.98	0.96	0.96	0.98	0.98	0.96	0.96	0.95	0.95	0.96	0.98
SSH6_AV1	1	1	0.99	1	1	1	1	1	0.98	0.98	1	1	0.98	0.98	0.97	0.97	0.98	
SSH6_AN1	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99	0.97	0.97	0.99	0.99	0.98	0.99	0.96	0.98		
SSH6_AKo1	0.98	0.98	0.97	0.98	0.98	0.98	0.98	0.98	0.97	0.97	0.98	0.98	0.96	0.96	0.96			
SSH6_AK24_2	0.98	0.98	0.97	0.98	0.98	0.98	0.98	0.98	0.97	0.97	0.98	0.98	0.96	0.97				
SSH6_AK24_1	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.99	0.99	0.98	0.98	0.97					
SSH6_AH1	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.97	0.97	0.98	0.98						
SSH6_AK9a_2	1	1	1	1	1	1	1	1	0.99	0.99	1							
SSH6_AK9a_1	1	1	1	1	1	1	1	1	0.99	0.99								
SSH6_AK2_2	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99	1									
SSH6_AK2_1	0.99	0.99	0.98	0.99	0.99	0.99	0.99	0.99										
SSH6_ACS_2	1	1	1	1	1	1	1											
SSH6_ACS_1	1	1	1	1	1	1												
SSH6_ACQ_2	1	1	1	1	1													
SSH6_ACQ_1	1	1	1	1														
SSH6_ACfz2_2	1	1	1															
SSH6_ACfz2_1	1	1																
SSH6_AC19_2	1																	

Table 4-4. The intra-specific and inter-specific pairwise similarities between SSH6 regions from *Aconitum* species

Species	Intra-specific pairwise similarity	Pairwise similarity with other species
A. carmichaeli	0.995-1	0.972-0.995
A. kusnezoffii	---	0.963-0.986
A. hemsleyanum	---	0.958-0.981
A. nagarum	---	0.963-0.986
A. vilmorinianum	0.981	0.954-0.995
A. coreanum	---	0.954-0.981

4.5 Results – SSH15

4.5.1 Sequence information

Sequence information was successfully obtained from 32 *Aconitum* samples. The sequence lengths ranged from 394bp to 400bp with an average of 397bp. The aligned length of SSH15 was 415bp. The average G+C content was 34.1%. In the aligned sequences, the number of in-del sites, variable sites and informative sites were 37, 188 and 77 respectively. Table 4-9 shows a detailed comparison of sequence information of SSH15 with other sequence markers. The aligned SSH15 of *Aconitum* is shown in Appendix E.

4.5.2 Sequence similarity

The pairwise sequence similarities between SSH15 from *Aconitum* samples are shown in Table 4-5. A summary of the ranges of intra-specific and inter-specific similarities are shown in Table 4-6.

Table 4-5. Pairwise sequence similarities between SSH15 regions from *Aconitum* samples

Sample	AC11_1	AC23_1	AC24_1	AC25_1	AC4_1	ACf2r2_3	ACf2r2_2	Acf2r3_1	ACQ_1	ACS_1	AK10_1	AK10_2	AK10_3	AK19_1	AK19_2	AK19_3	AK19_4	AK19_5	AK21_1	AK21_2	AK23_1	AK24_1	AK24_2	AK2_1	AK2_2	AK2_3	AK4_1	AK4_2	AK4_3	AK5_1	AK5_2	AK5_3	AK6c_1	AK8_1	AK9a_1	AK9b_1	AKf2f1_1	AKf2f1_2	AKf2f1_3	AKf2f2_1	AKf2f2_2	AKf2f2_3	AHI1_1	AHI_1	ANI_1	AV1_1	AV1_2	AV2_1	AV3_1	AV4_1	AKo1_1
AC11_1	0.96																																																		
AC23_1		0.96																																																	
AC24_1			0.94																																																
AC25_1				0.98																																															
AC4_1					0.93																																														
ACf2r2_3						0.93																																													
ACf2r2_2							0.94																																												
Acf2r3_1								0.94																																											
ACQ_1									0.97																																										
ACS_1										0.91																																									
AK10_1											0.9																																								
AK10_2												0.94																																							
AK10_3													0.96																																						
AK19_1														0.94																																					
AK19_2															0.96																																				
AK19_3																0.94																																			
AK19_4																	0.93																																		
AK19_5																		0.95																																	
AK21_1																			0.93																																
AK21_2																				0.98																															
AK23_1																					0.85																														
AK24_1																						0.97																													
AK24_2																							0.9																												
AK2_1																								0.9																											
AK2_2																									0.98																										
AK2_3																										0.94																									
AK4_1																											0.96																								
AK4_2																												0.97																							
AK4_3																													0.96																						
AK5_1																														0.94																					
AK5_2																															0.94																				
AK5_3																																0.97																			
AK6c_1																																	0.97																		
AK8_1																																		0.97																	
AK9a_1																																			0.92																
AK9b_1																																				0.91															
AKf2f1_1																																					0.94														
AKf2f1_2																																						0.96												
AKf2f1_3																																							0.95										
AKf2f2_1																																								0.94									
AKf2f2_2																																									0.95								
AKf2f2_3																																										0.92						
AHI1_1																																										0.93					
AHI_1																																											0.97			
ANI_1																																												0.97		
AV1_1																																													0.95	
AV1_2																																													0.96	
AV2_1																																														0.96
AV3_1																																														
AV4_1																																														
AKo1_1																																														

Table 4-6. The intra-specific and inter-specific pairwise similarities between SSH15 regions from *Aconitum* species

Species	Intra-specific pairwise similarity	Pairwise similarity with other species
A. carmichaeli	0.922-0.972	0.88-0.982
A. kusnezoffii	0.919-0.984	0.886-0.982
A. hemsleyanum	0.93	0.89-0.984
A. nagarum	---	0.888-0.982
A. vilmorinianum	0.949-0.979	0.885-0.984
A. coreanum	0.957	0.88-0.922

4.5.3 Phylogram study

Phylogram study of SSH15 of *Aconitum* using neighbor-joining (NJ), unweighted pair-group method using arithmetic mean (UPGMA) and maximum parsimony (MP) with bootstrap tested by 1000 replicates was carried out. Data are shown in Figure 4-2, Figure 4-3 and Figure 4-4 respectively.

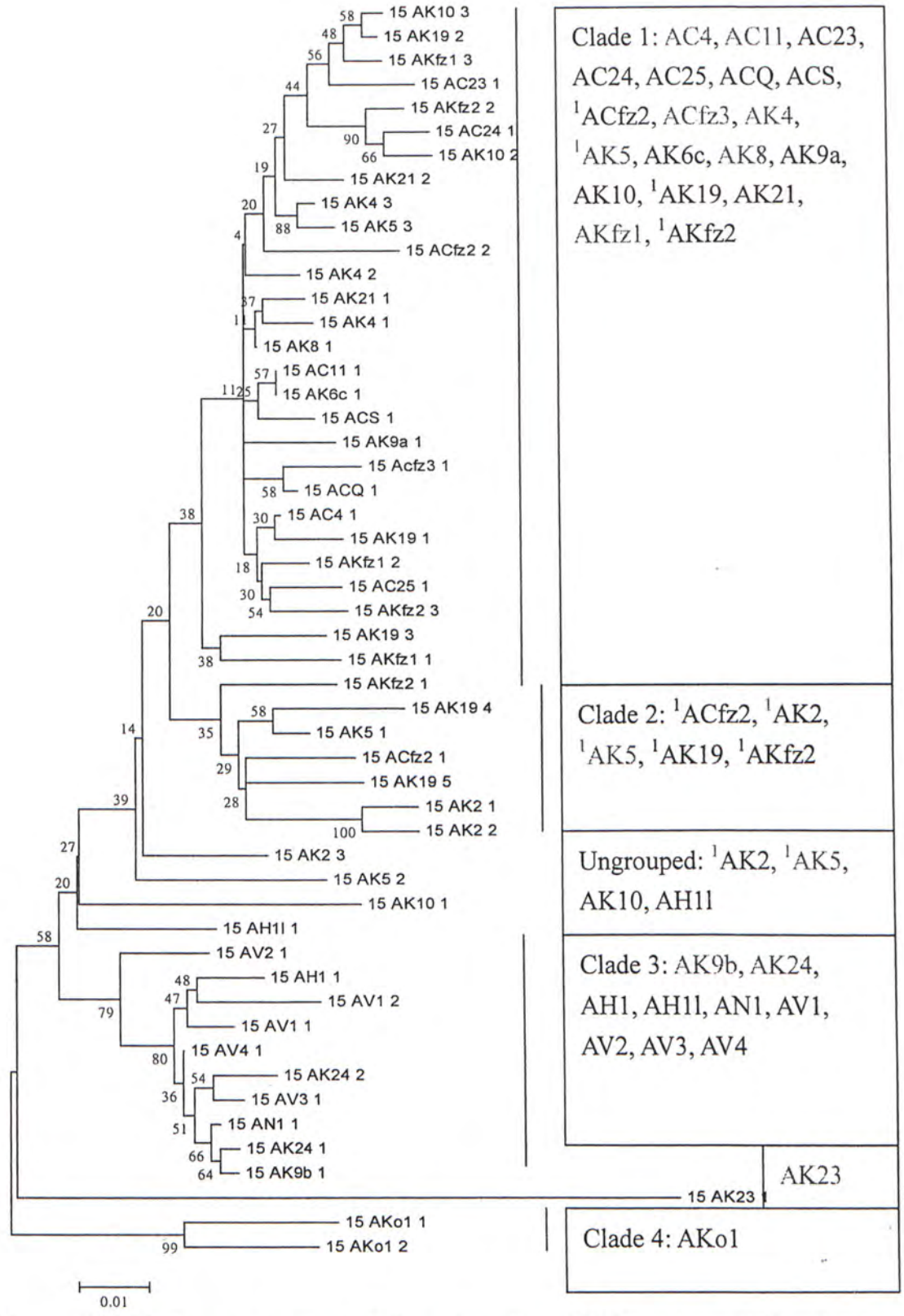

Figure 4-2. Phylogram constructed from *Aconitum* SSH15 using neighbor-joining method with bootstrap tested by 1000 replicates. Sample codes are listed on right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents different sequences from one sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

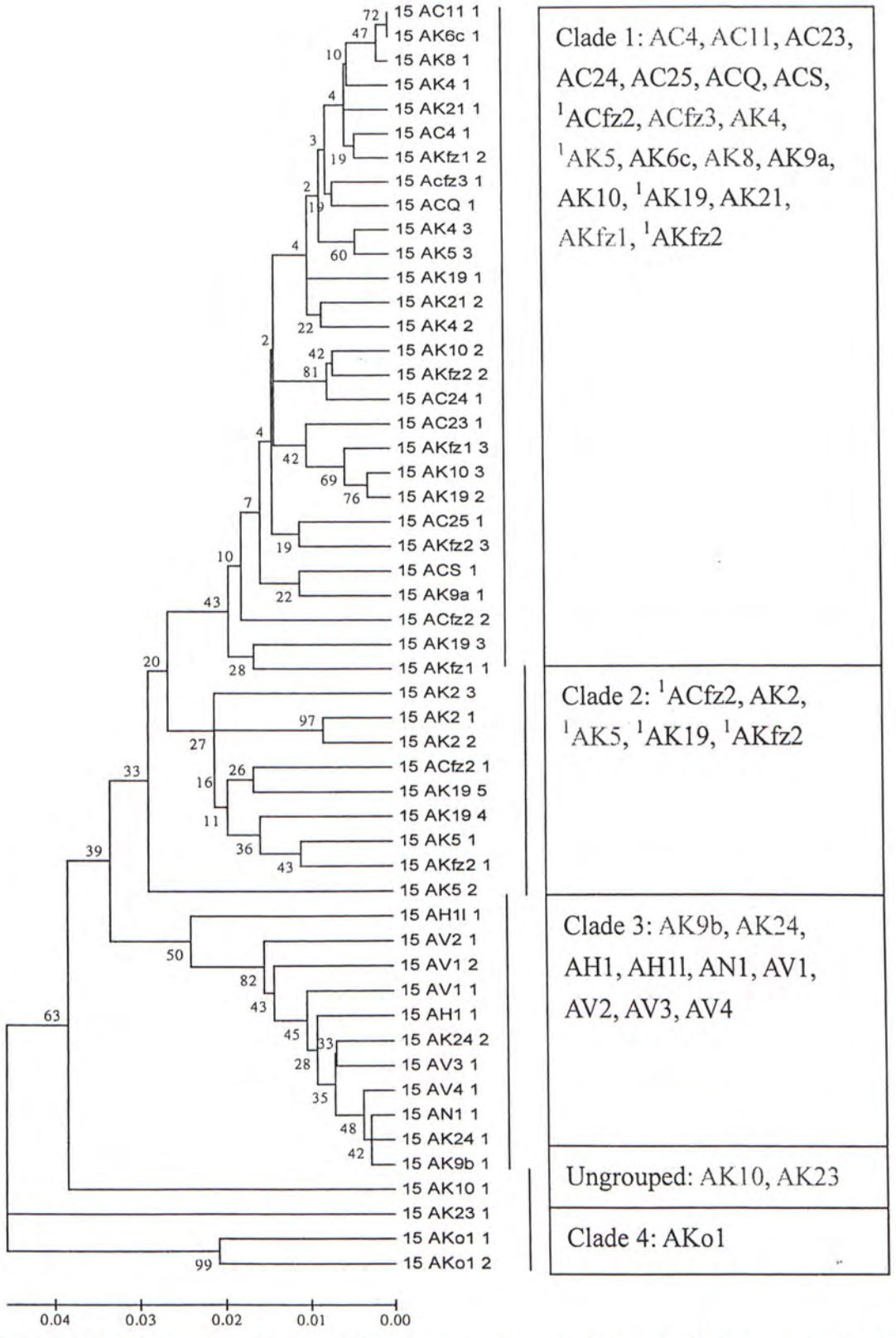

Figure 4-3. Phylogram constructed from *Aconitum* SSH15 by UPGMA method with bootstrap tested by 1000 replicates. Sample codes are listed on right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents different sequences from one sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

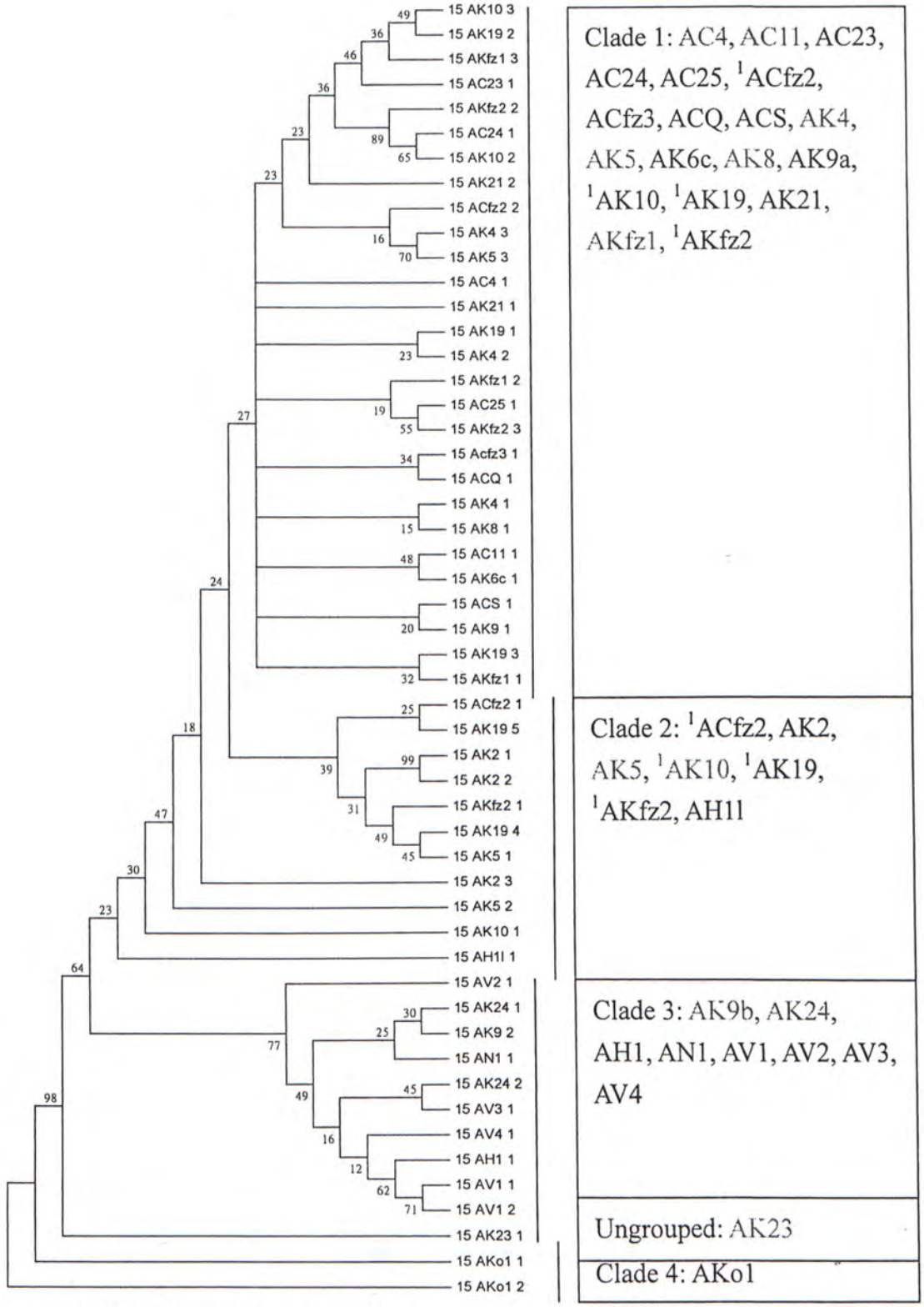

Figure 4-4. Phylogram constructed from *Aconitum* SSH15 by maximum parsimony method with bootstrap tested by 1000 replicates. Samples which are involved in clades are listed on right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents any samples which occur in more than one clade. Samples with unknown identity are shaded in grey.

4.6 Results – SSH45

4.6.1 Sequence information

Sequence information was successfully obtained from 15 *Aconitum* samples. The sequence lengths ranged from 352bp to 369bp with an average of 365bp. The aligned length of SSH45 was 377bp. The average G+C content was 34.9%. In the aligned sequences, the number of in-del sites, variable sites and informative sites were 35, 162 and 76 respectively. Table 4-9 shows a detailed comparison of sequence information of SSH45 with other sequence markers. The aligned SSH45 of *Aconitum* is shown in Appendix F.

4.6.2 Sequence similarity

The pairwise sequence similarities between SSH45 from *Aconitum* samples are shown in Table 4-7. A summary of the ranges of intra-specific and inter-specific similarities are shown in Table 4-8.

Table 4-7. Pairwise sequence similarities between SSH45 regions from *Aconitum* samples

Sample	AC19_2	ACfz2_1	ACfz2_2	ACS_1	ACS_2	AK10_1	AK10_2	AK10_3	AK19_1	AK19_2	AK19_3	AK21_1	AK21_2	AK21_3	AK21_4	AK24_1	AK2_1	AK5_1	AKfz1_1	AKfz1_2	AKfz1_3	AKfz2_1	AKfz2_2	AKfz2_3	AH1_1	AN1_1	AV1_1	AKo1_1
AC19_1	0.942	0.901	0.945	0.887	0.882	0.898	0.864	0.923	0.967	0.896	0.927	0.945	0.950	0.879	0.959	0.883	0.896	0.928	0.879	0.939	0.953	0.827	0.896	0.931	0.898	0.887	0.887	0.882
AC19_2		0.891	0.923	0.872	0.869	0.888	0.857	0.915	0.942	0.883	0.927	0.923	0.929	0.866	0.929	0.870	0.894	0.907	0.872	0.918	0.932	0.825	0.883	0.910	0.896	0.885	0.875	0.872
ACfz2_1			0.912	0.947	0.953	0.909	0.937	0.891	0.896	0.912	0.889	0.907	0.907	0.950	0.894	0.942	0.967	0.909	0.953	0.923	0.909	0.909	0.961	0.898	0.975	0.958	0.945	0.904
ACfz2_2				0.904	0.898	0.912	0.886	0.920	0.945	0.918	0.913	0.961	0.961	0.896	0.932	0.899	0.912	0.939	0.901	0.964	0.961	0.846	0.912	0.948	0.915	0.907	0.901	0.893
ACS_1					0.950	0.898	0.923	0.888	0.890	0.909	0.870	0.904	0.898	0.947	0.880	0.926	0.942	0.907	0.953	0.915	0.901	0.898	0.964	0.901	0.939	0.939	0.928	0.906
ACS_2						0.901	0.934	0.888	0.882	0.904	0.875	0.893	0.893	0.964	0.880	0.931	0.947	0.901	0.956	0.909	0.896	0.909	0.969	0.896	0.945	0.939	0.934	0.898
AK10_1							0.886	0.882	0.898	0.907	0.881	0.912	0.912	0.898	0.885	0.907	0.904	0.901	0.907	0.923	0.915	0.857	0.915	0.909	0.901	0.898	0.909	0.888
AK10_2								0.864	0.864	0.897	0.860	0.891	0.880	0.945	0.862	0.918	0.929	0.886	0.931	0.897	0.888	0.877	0.942	0.883	0.920	0.923	0.926	0.877
AK10_3									0.918	0.896	0.916	0.926	0.915	0.880	0.921	0.886	0.896	0.920	0.885	0.926	0.923	0.831	0.896	0.929	0.893	0.893	0.896	0.877
AK19_1										0.896	0.916	0.948	0.950	0.879	0.959	0.886	0.896	0.928	0.879	0.939	0.953	0.830	0.896	0.931	0.898	0.887	0.887	0.882
AK19_2											0.878	0.918	0.923	0.907	0.888	0.899	0.912	0.915	0.907	0.939	0.920	0.852	0.923	0.926	0.904	0.901	0.907	0.893
AK19_3												0.908	0.913	0.878	0.919	0.884	0.889	0.908	0.867	0.913	0.921	0.821	0.883	0.900	0.891	0.881	0.889	0.872
AK21_1													0.967	0.896	0.932	0.894	0.912	0.950	0.896	0.961	0.969	0.838	0.907	0.953	0.909	0.912	0.907	0.899
AK21_2														0.890	0.937	0.899	0.907	0.945	0.901	0.961	0.969	0.841	0.907	0.959	0.909	0.901	0.901	0.893
AK21_3															0.877	0.929	0.950	0.898	0.950	0.907	0.898	0.901	0.967	0.893	0.942	0.942	0.936	0.895
AK21_4																0.881	0.894	0.921	0.872	0.932	0.940	0.823	0.888	0.929	0.899	0.885	0.885	0.875
AK24_1																	0.937	0.899	0.934	0.913	0.897	0.885	0.940	0.897	0.934	0.929	0.961	0.899
AK2_1																		0.909	0.950	0.923	0.915	0.898	0.956	0.904	0.980	0.980	0.939	0.901
AK5_1																			0.904	0.950	0.948	0.844	0.915	0.942	0.907	0.904	0.901	0.896
AKfz1_1																				0.912	0.898	0.909	0.967	0.898	0.945	0.939	0.936	0.898
AKfz1_2																					0.969	0.860	0.923	0.964	0.926	0.918	0.915	0.910
AKfz1_3																						0.841	0.909	0.950	0.912	0.909	0.901	0.891
AKfz2_1																							0.912	0.838	0.898	0.895	0.884	0.852
AKfz2_2																								0.909	0.953	0.947	0.942	0.912
AKfz2_3																									0.901	0.898	0.898	0.891
AH1_1																										0.972	0.936	0.898
AN1_1																											0.936	0.898
AV1_1																												0.906

Table 4-8. The intra-specific and inter-specific pairwise similarities between SSH45 regions from *Aconitum* species

Species	Intra-specific pairwise similarity	Pairwise similarity with other species
A. carmichaeli	0.869-.0953	0.866-0.975
A. kusnezoffii	0.877-0.967	0.866-0.98
A. hemsleyanum	---	0.891-0.98
A. nagarum	---	0.881-0.98
A. vilmorinianum	---	0.875-0.945
A. coreanum	---	0.872-0.906

4.6.3 Phylogram study

Phylogram study of SSH45 of *Aconitum* using three methods with bootstrap tested by 1000 replicates: neighbor-joining (NJ), unweighted pair-group method using arithmetic mean (UPGMA) and maximum parsimony (MP) was carried out. Data are as shown in Figure 4-5, Figure 4-6 and Figure 4-7 respectively.

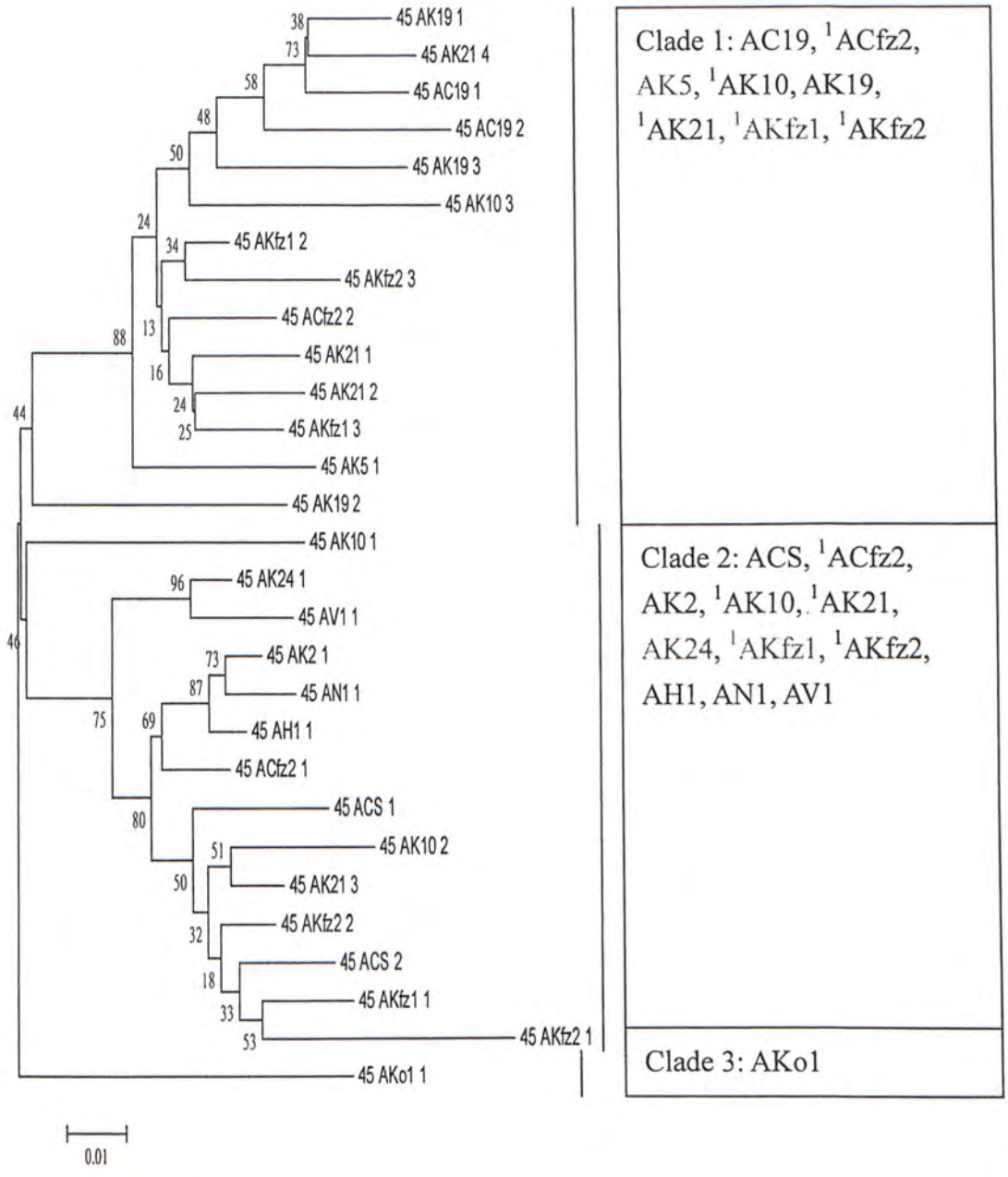

Figure 4-5. Phylogram constructed from *Aconitum* SSH45 using neighbor-joining method with bootstrap tested by 1000 replicates. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents different sequences from one sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

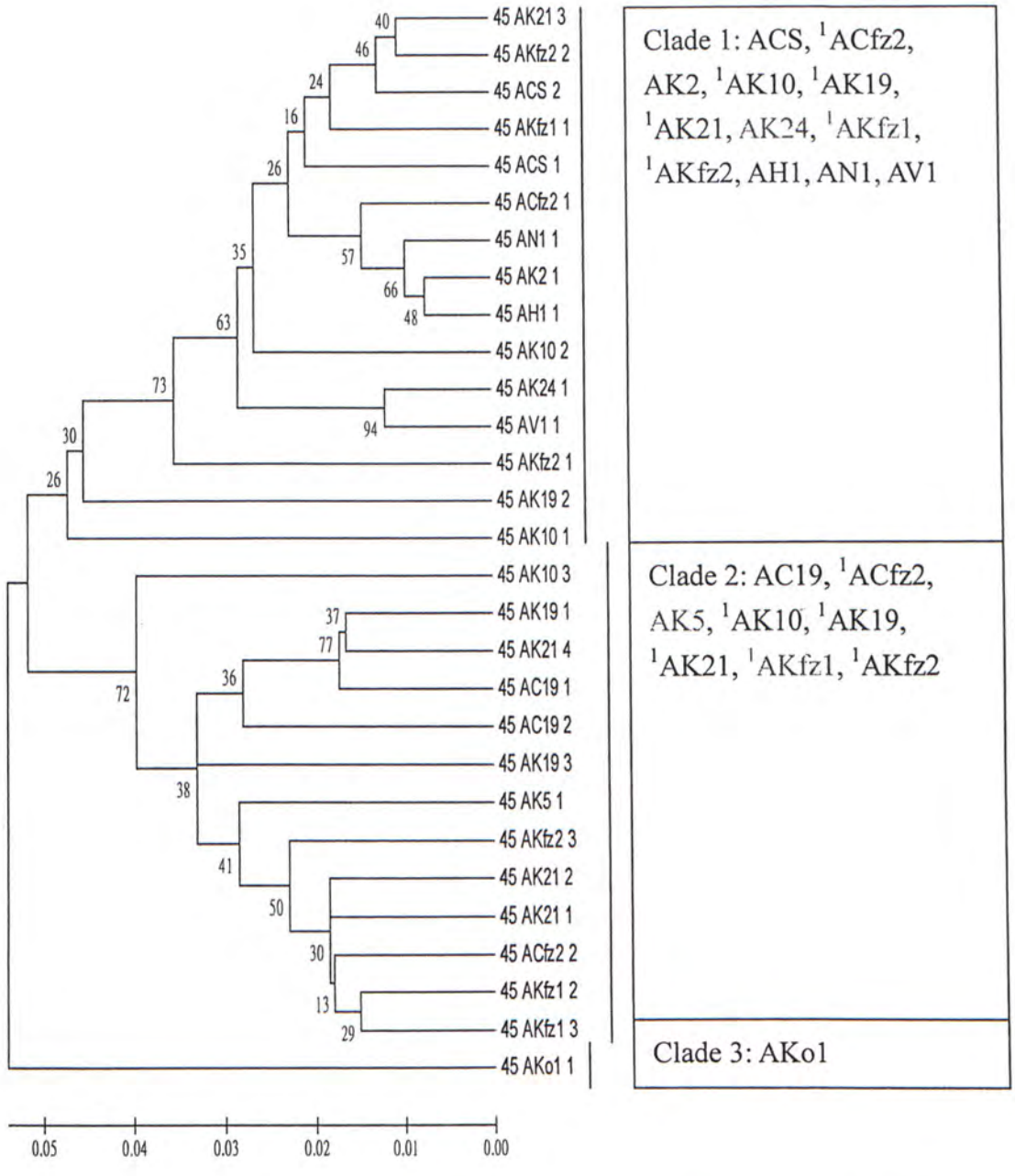

Figure 4-6. Phylogram constructed from *Aconitum* SSH45 using UPGMA method with bootstrap tested by 1000 replicates. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents different sequences from one sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

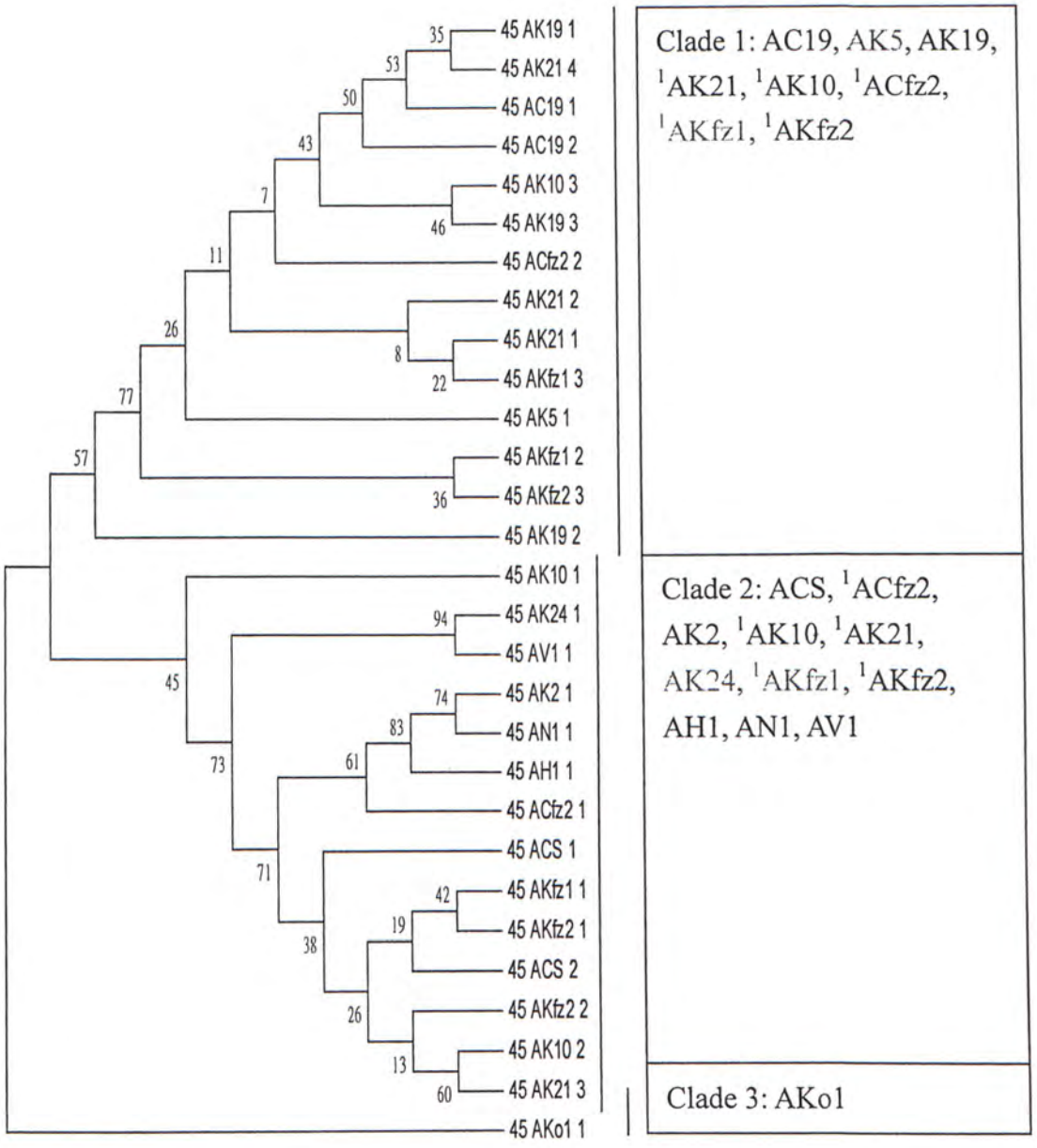

Figure 4-7. Phylogram constructed from *Aconitum* SSH45 using maximum parsimony method with bootstrap tested by 1000 replicates. Sample codes are listed on the right, AC: *A. carmichaeli*, AK: *A. kusnezoffii*, AH: *A. hemsleyanum*, AN: *A. nagarum*, AV: *A. vilmorinianum*, AKo: *A. coreanum*, AS: *Aconitum sp.*. [1] represents different sequences from one sample which occur in more than one clade. Samples with unsure identities are shaded in grey.

4.7 Discussion

Table 4-9. A comparison of commonly-used and screened markers

DNA Region	Raw length /bp (Average)	Aligned length /bp	Average G+C%	Indel sites (%)	Variable sites (%)	Informative sites (%)
5S spacer	577-660 (628)	725	46.9	342 (47.2)	635 (87.6)	486 (67.0)
*psb*A-*trn*H spacer	222-239 (230)	264	31.5	53 (20.1)	58 (22.0)	26 (9.8)
SSH6	213-216 (215)	218	36.8	9 (4.1)	12 (5.5)	2 (0.9)
SSH15	394-400 (397)	415	34.1	37 (8.9)	188 (45)	77 (18.5)
SSH45	352-369 (365)	377	34.9	35 (9.3)	162 (42.9)	76 (20.1)

4.7.1 Utility of subtraction in screening markers

The use of genomic subtraction successfully led to several differential clones between two *Aconitum* samples, and the clones can detect relatively high degree of polymorphism between individuals. The percentage of variable sites of two of the clones, SSH15 and SSH45, were up to 40%, which was even higher than a commonly-used molecular marker *psb*A-*trn*H spacer (22%) (Table 4-9). The numbers of informative sites of SSH15 and SSH45 were also higher than those of *psb*A-*trn*H spacer. What makes them more favorable as molecular markers is that they had relatively few in-del sites. That means they had nearer raw lengths to the

aligned lengths (as compared to another commonly-used molecular marker such as the 5S spacer), making it simpler and more objective in aligning the sequences.

The capability of genomic subtraction to screen for variations between genomes is also shown in two of the clones, SSH3 and SSH5. They had BLASTN results matched with *Aconitum* microsatellites, which are variable repeats that scatter throughout the genome. Another two clones, SSH37 and SSH42, also gave BLASTN results which belong to the same family with *Aconitum*, showing that genomic subtraction can also screen for markers which can differentiate *Aconitum* at a low taxonomic levels.

In genomic subtraction, some clones, SSH17, SSH25, SSH27, SSH34, SSH46 and SSH49, did not give any match in BLASTN search. They may be other good starting points to screen for novel markers for authentication of *Aconitum*.

In theory, genomic subtraction can screen for differential clones which exist in one genome but not the other. However, in the authentication of TCM, these differential clones are not useful as markers. Markers which can be amplified in one species but not another are purposely excluded for the following reason. In dealing with

CMMs, many of their genomes are damaged or destroyed, making it virtually impossible to give PCR products. Therefore, if such markers cannot be amplified in certain samples, there are still two possibilities: (1) the sample is an adulterant or (2) the DNA quality is poor. Therefore in this project, the focus is on markers which give sequence information in most *Aconitum* species.

4.7.2 SSH6

SSH6 was a conserved marker across genus *Aconitum*, and this could be seen from the high similarities between different *Aconitum* species (Table 4-3). There were only 12 variable sites out of 218bp alignment. Despite its conserved sequences, it was the only marker which can clearly differentiate *A. carmichaeli* from *A. kusnezoffii* by 2 variable sites at positions 72 and 183 (Figure 4-8). It could also be used to identify *A. vilmorinianum* by the variable site at position 74 on the alignment.

SSH6 was distinctive from other markers as its variation among *Aconitum* species was the lowest. However, this characteristic gave us consistent results and is essential in authentication as *Aconitum* genome seems to be highly variable as shown in the phylogram study of other markers such as 5S spacer, *psb*A-*trn*H spacer and SSH15.

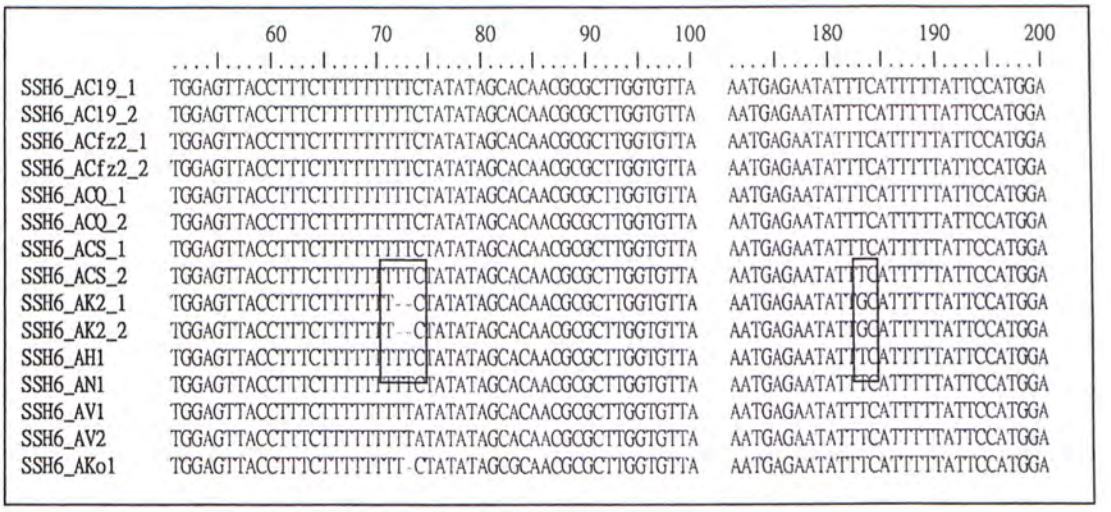

Figure 4-8. A region of SSH6 which can differentiate *A. carmichaeli* and *A. kusnezoffii*. Variable sites at position 72 and 183 (highlighted) can differentiate *A. kusnezoffii* from other *Aconitum* species.

4.7.3 SSH15

SSH15 was a marker which was variable across genus *Aconitum* and almost half of the sequence contained variable sites. Phylogram studies from the three methods supported the grouping of *Aconitum* into four clades.

Clade 4 from the three phylograms (Figure 4-2, Figure 4-3 and Figure 4-4) consisted of only *A. coreanum*. Together with the evidence from 5S spacer and *psb*A-*trn*H spacer, it implied *A. coreanum* could be well distinguished from the rest of *Aconitum* species. The similarity study further supported this as the intra-specific similarity of SSH15 from *A. coreanum* was higher than its inter-specific similarity.

The grouping of Clades 1 and 2 was only weakly supported by low bootstrap values in all three phylogram studies (Figure 4-2, Figure 4-3 and Figure 4-4). In both Clades 1 and 2, only *A. carmichaeli* and *A. kusnezoffii* could be found. Although there were more clones of *A. carmichaeli* which existed in Clade 1 and of *A. kusnezoffii* in Clade 2, some samples had clones in both Clades 1 and 2: ACfz2 and AK21. This revealed a close relationship between *A. carmichaeli* and *A. kusnezoffii* which was also shown in the phylogram studies of 5S spacer and *psb*A-*trn*H spacer.

Clade 3 (Figure 4-2, Figure 4-3 and Figure 4-4) was well-separated from the other clades in the three phylogram studies, and it consisted of *A. hemsleyanum*, *A. nagarum* and *A. vilmorinianum*. In Clade 3, non-Pharmacopoeia-listed *Aconitum* species could be identified which was also be supported by phylogram studies in 5S spacer and *psb*A-*trn*H spacer.

4.7.4 SSH45

SSH45 had a similar percentage of variable sites as SSH15. In phylogram study of SSH45, sample AKo1 (*A. coreanum*) had been set as an out-group which was revealed previously in 5S spacer and *psb*A-*trn*H spacer. The remaining samples formed two clades in all the three methods including NJ, UPGMA and MP (Figure

4-5, Figure 4-6 and Figure 4-7). Clones from five of the samples clustered in both clades including ACfz2, AK10, AK21, AKfz1 and AKfz2. Samples of *A. carmichaeli* and *A. kusnezoffii* clustered in both clades but samples of non-Pharmacopoeia-listed species are only found in Clade 2, including *A. hemsleyanum*, *A. nagarum* and *A. vilmorinianum*.

The situation that clones from some samples were clustered in two separate clades strongly suggest that those samples are hybridization products. SSH45 provided strong evidence, together with 5S spacer, for the occurrence of hybridization for sample *Aconitum* species.

4.7.5 Hybridization in *Aconitum*

Authentication of non-Pharmacopoeia-listed species can be archived by three markers: 5S spacer, *psb*A-*trn*H and SSH15. However, identifying *A. carmichaeli* and *A. kusnezoffii* is difficult due to their close phylogenetic relationship. So far, only SSH6 can provide a consistent 2-bp deletion in *A. kusnezoffii* and the deletion can be used to identify *A. kusnezoffii* from *A. carmichaeli*.

A. carmichaeli and *A. kusnezoffii* do show a complicated relationship with other

Aconitum species, which shows that the hybridization was involved in the evolution of the two species. Both *A. carmichaeli* and *A. kusnezoffii* belong to Ser. Inflata which involves polyploidy *Aconitum* species. Recent phylogenetic studies show that *Aconitum volubile* (Ser. Volubilia) which is a diploid species were often clustered with Ser. Inflata (Luo *et al.*, 2005; Kita and Ito, 2000). In this project, species of Ser. Volubilia, including *A. hemsleyanum* and *A. vilmorinianum* were also studied. They also showed a relatively close relationship (especially revealed by SSH45) with species from Ser. Inflata. This supports the hypothesis of Luo *et al.* and Kita and Ito that Ser. Inflata and Ser. Volubilia may have a common ancestor. Ser. Inflata which includes *A. carmichaeli* and *A. kusnezoffii* can be resulted from complicated hybridization of its early ancestors.

Complication in hybridization in *A. carmichaeli* and *A. kusnezoffii* makes authentication difficult since DNA from other species can also exist in the genomes of the two Pharmacopoeia-listed species. In the three markers screened, only SSH6 can identify *A. kusnezoffii* from the rest, which still shows the usefulness of genomic subtraction to screen for markers for complicated groups.

4.7.6 Inferring species identities of samples from the market

Samples from the market were authenticated by sequence markers: 5S spacer, *psb*A-*trn*H spacer and SSH15. The species identities were interpreted from clades in which the samples were clustered and the interpretation of each region is shown in Table 4-10.

Authentication of Aconitum by the three regions (5S spacer, *psb*A-*trn*H spacer and SSH15) showed consistent results (Table 4-10). In 12 samples, more than one region were sequenced and interpretations from different regions were consistent. Their final interpretations were considered with higher confidence.

In the final interpretation, of the 17 samples which claimed to be Pharmacopoeia-listed species, 8 (47%) were unlisted *Aconitum* species. This revealed that the adulteration of *Aconitum* in TCM has become a serious problem.

Table 4-10. Interpretation of identities of samples from the market. The table shows whether the samples are Pharmacopoeia-listed *Aconitum* species or not. Blank means no sequence data obtained. #Interpreted from only one region.

Sample	Marker used for Interpretation			Final Interpretation
	5S spacer	*psb*A-*trn*H spacer	SSH15	
AC1		Listed		Listed #
AC3	Unlisted	Unlisted		Unlisted
AC4		Listed	Listed	Listed
AC5	Medicinal			Listed #
AC7		Unlisted		Unlisted #
AC11		Listed	Listed	Listed
AC12		Unlisted		Unlisted #
ACfz3	Listed		Listed	Listed
AK4		Listed	Listed	Listed
AK5	Listed	Listed	Listed	Listed
AK7	Unlisted	Unlisted		Unlisted
AK8	Listed	Listed	Listed	Listed
AK9b	Unlisted	Unlisted	Unlisted	Unlisted
AK11		Unlisted		Unlisted #
AK23		Unlisted	Unlisted	Unlisted
AK24	Unlisted	Unlisted	Unlisted	Unlisted
AKfz1		Listed	Listed	Listed
AS2	Listed			Listed #
AS3	Unlisted			Unlisted #
AS4	Unlisted			Unlisted #
ASfz1	Listed			Listed #
ASfz2	Listed			Listed #
ASfz3		Listed		Listed #

4.8 Conclusion

It is suspected that hybridization has taken an important part in the evolution to *Aconitum* (Luo *et al.*, 2005; Kita and Ito, 2000) as inferred by the analysis of different sequence markers in this project. Although hybridization makes it difficult to identify *A. carmichaeli* from *A. kusnezoffii*, it is still possible to distinguish Pharmacopoeia-listed *Aconitum* species from the unlisted ones.

Twenty-two samples were collected on the markets from Mainland and Hong Kong and analyzed by commonly used phylogenetic markers and markers which were screened from genomic subtraction. Ten of the samples were non-Pharmacopoeia-listed species. Guo and Jia (1990) also reported the availibility of several non-Pharmacopoeia-listed species (*A. hemsleyanum* and *A. legendrei*) on the medicinal market. These indicated the seriousness of problem of the adulteration of *Aconitum*. Authentication by sequencing offers an effective means to distinguish Pharmacopoeia-listed *Aconitum* species from the unlisted ones. This technique should be further applied in routine inspection along the supply line of CMMs in order to ensure public safety.

Chapter 5. Assessment of *Aucklandia lappa* and Related Species by GC-MS

5.1 Introduction

In this chapter, authentication and quality assessment by chemical method are described. Gas chromatography- mass spectrometry was used to study the essential oil content of *Aucklandia lappa*, *Inula helenium*, *Vladimiria souliei* and other related species. The three species belong to Asteraceae, and their root parts are used in TCM with the same suffix, Muxiang, in their Chinese names.

Their essential oil is pharmacologically active (Section 1.12.2) and therefore the oil content is the focus of this study. The essential oil was extracted and analyzed by GC-MS to generate a chemical profile for each root sample. Then the profiles were compared by a chemometric method called hierarchical cluster analysis, which allows discriminating one profile from another with the help of mathematical methods. Chemical markers of *A. lappa* were also extracted and standardized by GC-MS, and their contents in *A. lappa* were determined.

5.2 Methods

Medicinal materials tested are listed in Section 2.1.2. Essential oil of each root sample (50g) was extracted by distillation as described in Section 2.3. For the extraction, each oil sample was analyzed by GC-MS to generate a chemical profile as described in Section 2.8. The profiles were then processed by computer software to make a comparison among them according to Section 2.9.

Chemical markers were extracted and purified by using the method in Section 2.4. Serial dilutions of the markers were also analyzed by GC-MS as described in Section 2.8. Standardization curves were generated by plotting peak abundance against mass injected into the GC system. The content of the chemical markers in each *A. lappa* sample were then determined from the standardization curves. NIST98 Mass Spectral Library was used to assist in aligning peaks and identifying the chemicals.

5.3 Results

5.3.1 Extraction of essential oil

Essential oil from root parts of *Aucklandia lappa* and related species was extracted

successfully from 27 samples of *Aucklandia lappa*, 9 samples of *Inula helenium*, 3 samples of *Inula racemosa*, 3 samples of *Vladimiria berardioidea*, 12 samples of *Vladimiria souliei* and 10 samples of *Vladimiria souliei* var. *cinerea*. The amount of essential oil recovered ranged from 0.090mg to 0.508mg with an average of 0.255mg, which means 0.18% to 1.016% with an average 0.51%(w/w) from 50g root materials.

5.3.2 GC-MS analysis

Essential oil from all the extracted samples was analyzed by GC-MS. Figure 5-1 shows a combined and aligned chromatogram from selected samples from each species. Individual GC profile of each sample can be found in Appendix G.

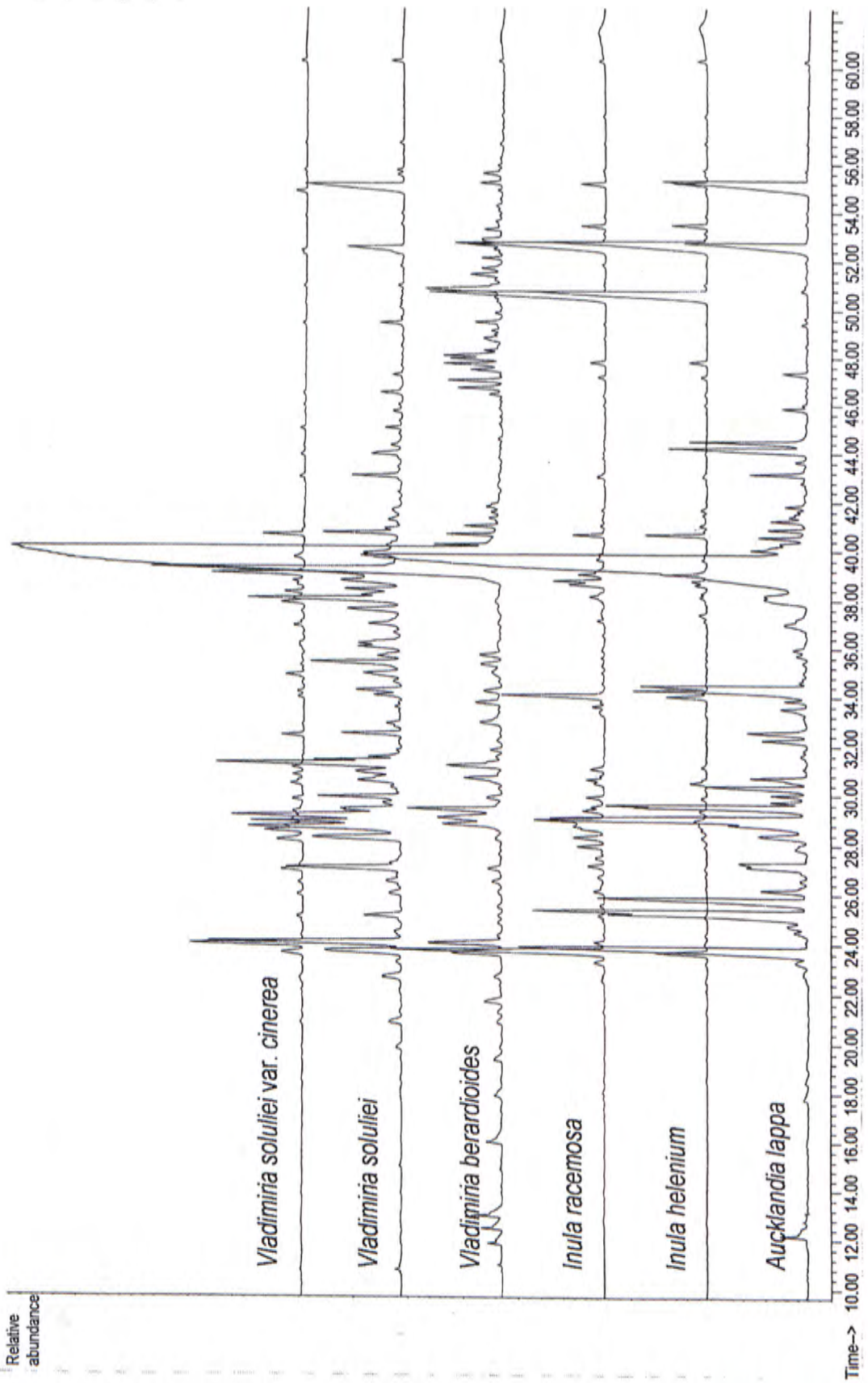

Figure 5-1. A combined and aligned chromatogram from *Aucklandia lappa* and related species. Sample AL23, IH07, IR05, VB03, VS17 and VSV02 are shown here for illustration.

5.3.3 Peak alignment and hierarchical cluster analysis

12 samples of *Aucklandia lappa*, 9 samples of *Inula helenium*, 3 samples of *Inula racemosa*, 3 samples of *Vladimiria berardioidea*, 12 samples of *Vladimiria souliei* and 10 samples of *Vladimiria souliei* var. *cinerea* were chosen for hierarchical cluster analysis.

Peaks from a sample were converted to area percentages, and peaks from different samples were aligned according to their peak identities. The percentage peak area matrix is shown in Table 5-1. The top 256 peaks, according to the total percentage area, were selected for hierarchical cluster analysis. A list of chemicals detected in *A. lappa* is shown in Table 5-3.

Pairwise relationships were calculated by both distance and similarity method: square Euclidean distance and cosine of vectors. The values are listed in Table 5-2. Dendrograms were constructed with the use of the two calculation methods mentioned in Section 1.16.4. The dendrogram calculated by square Euclidean distance method with between-groups linkage is shown in Figure 5-2 and that calculated by cosine vector with between-groups linkage is shown in Figure 5-3.

Table 5-1. Percentage peak area matrix. Undetectable peaks are shaded.

R.T / Sample	10.75	10.78	10.87	12.18	12.95	18.50	19.39	19.80	20.81	21.21	21.26	21.82	22.13	22.53	22.62	22.79	23.22	23.70	24.00	24.34	24.44
AL1				0.06		0.04										0.04	0.45	7.57			0.12
AL11				0.14		0.04										0.15	0.06	2.43			0.11
AL12				0.02		0.05											0.09	2.85			0.22
AL15				0.09		0.03										0.04	0.07	2.18			0.21
AL16				0.19		0.01										0.10	0.15	5.32			0.20
AL17				0.10		0.01										0.12	0.42	8.27			0.16
AL21				0.25		0.09										0.22	0.32	6.74			0.26
AL22				0.10		0.06										0.05	0.12	3.47			
AL23				0.09		0.01										0.10	0.17	4.25			0.11
AL25				0.23		0.04										0.17	0.14	3.16			0.61
AL25a				0.09												0.08	0.08	2.78			0.36
AL28				0.18		0.06										0.22	0.15	4.66			0.69
IH02																0.09		0.07	1.45	0.23	0.09
IH07						0.17	0.13				0.20			0.26			1.04	14.86		0.08	
IH08							0.13							0.30			0.35	5.75	2.34		
IH11							0.09				0.19			0.14			0.25	5.22	1.11	0.10	
IH12											0.30			0.23			0.74	12.38	0.30	0.19	
IH13														0.23			0.47	8.61	0.83	0.18	
IH16							0.12				0.19			0.27			0.24	5.00	0.25		
IH17						0.30	0.25				0.40			0.45			0.35	6.14	0.18		
IH18							0.11				0.27			0.34			0.54	11.84	0.49		
IR01														0.19				1.04		0.17	
IR03														0.33			0.78	16.02	0.61		
IR05														0.16			0.60	10.87	0.11	0.04	
VB01					0.17					0.42				1.12					0.21		
VB03				0.69			0.06	0.13	0.12					0.06	0.24		1.22		1.60		
VB05				0.76					0.30					0.12	0.07		2.59		0.23		
VS02	0.08		0.07					0.13	0.34		0.06	0.04		0.58		0.10		2.00	10.10		
VS07	0.02	0.16	0.09				0.13	0.38	0.09		0.06	0.17		0.63				7.71	14.31		
VS08	0.04	0.26	0.22				0.23	0.59		0.15		0.06		0.88					24.48		
VS09	0.03	0.11					0.20	0.48				0.11		0.76		0.14		2.73	2.75		
VS10	0.04	0.41	0.28				0.29	0.66	0.06		0.05			0.94				5.57	11.96		
VS12	0.02	0.32	0.22				0.28	0.59	0.08		0.04			0.94		0.19		2.66	4.18		
VS13	0.01	0.09	0.08				0.16	0.42	0.03		0.06	0.08		0.69		0.12		3.25	9.53		
VS15	0.09		0.08				0.13	0.41	0.13		0.21			0.67				11.76	17.13		
VS17	0.16						0.19	0.43	0.07			0.12		0.78		0.20	2.73	5.88	0.30		
VS18	0.01	0.06	0.03				0.12	0.30			0.06			0.22		0.25	0.09	2.83	10.93		
VS19	0.02	0.24	0.21				0.18	0.42	0.03		0.06	0.03		0.06		0.70	0.19	4.53	10.57		
VS20	0.03							0.16						0.34			0.85	5.27	0.08		
VSV02	0.18							0.39				0.18		0.94				3.33	16.72		
VSV03	0.02	0.30	0.22				0.32	0.75			0.08			0.96					23.66		
VSV04	0.25						0.20	0.39						0.81		0.19	3.84	0.60	0.20		
VSV06	0.03							0.20						0.27		0.14	3.06	6.21			
VSV07											0.26			0.75			3.63	7.19	0.41		
VSV10	0.02	0.09						0.29				0.05		0.43		0.07	2.03	0.65			
VSV11	0.01	0.05	0.04				0.10	0.28				0.12		0.46		0.08		1.81	7.88		
VSV18	0.03	0.16	0.09				0.18	0.41			0.05	0.08		0.68		0.12	2.07		5.52		
VSV19	0.08						0.05	0.17				0.30		0.06		0.10	2.05		10.13		
VSV20	0.06		0.05				0.10	0.25				0.04		0.45		0.09	1.07	0.62	5.49		

R.T / Sample	24.87	25.08	25.15	25.27	25.28	25.56	25.73	25.99	26.07	26.24	26.66	26.77	27.11	27.12	27.19	27.43	27.87	27.92	28.24	28.30	28.57
AL1		1.76	3.09				1.47		0.55				1.12	0.63			0.15			0.46	
AL11		0.17	2.80		1.52		2.85		0.65				2.03	0.05			0.19			0.60	
AL12		0.26	2.95				0.62		0.48				0.82				0.13			0.49	
AL15		2.03	1.86				1.99		0.56				1.67	0.06			0.16			0.63	
AL16		2.52	2.27				1.99		0.90				1.45	0.26						0.79	
AL17		4.81	3.85				1.96		1.29				1.32	0.95			0.25			0.86	
AL21		0.46	3.05		3.80		3.89		0.78				0.93	1.01			0.36			1.00	
AL22		0.27	3.44		2.49		1.65		0.49				0.91	0.86					0.13	0.62	
AL23		0.29	2.64		2.73		2.17		0.49				0.88	0.73			0.25			0.67	
AL25		0.37	3.59		3.39		2.32		1.20				1.08	1.24			0.28			1.27	
AL25a		2.35	2.14				1.64		0.67				0.64	0.82			0.18			0.89	
AL28		0.26	3.41		3.47		1.97		1.06				1.01	0.78			0.24			1.03	
IH02		0.14							0.12				0.10						0.13	0.27	0.10
IH07		1.51									1.00	0.52	0.91				0.25		1.52	2.10	
IH08		0.29							0.15	0.27	0.25		0.39			0.12			0.36	0.77	
IH11		0.45							0.14	0.29	0.28		0.58			0.09			0.61	0.95	1.31
IH12		0.39								0.48	0.44		0.14						0.65	1.23	
IH13		0.67							0.14	0.28	0.46		0.61			0.18			0.58		1.18
IH16		0.33								0.24	0.19		0.32						0.31	0.65	0.55
IH17		0.50							0.35	0.22									0.41		0.77
IH18		1.34							0.73	0.47			0.58	0.35		0.15			1.10	2.19	
IR01																			0.19	0.30	
IR03		0.40							0.68	0.34	0.62		1.13			0.63			1.61	2.23	1.23
IR05		0.35							0.20	0.33	0.27		0.54			0.13			1.57	2.05	
VB01									0.07												
VB03		0.10							0.14				0.38								
VB05		0.28							0.15				0.09						0.34		
VS02	0.12	0.19		0.75		0.07		0.67			0.69	0.13	2.58			0.55			0.10	3.40	
VS07	0.20	0.49		0.83		0.07		1.36			1.46		2.61			0.86			0.05	1.66	
VS08	0.04	0.55		0.98		0.06		1.51			1.65		3.80			0.88			0.06		
VS09				1.15		0.08		0.21				0.48	0.11	3.67		0.24			0.06		
VS10	0.11	0.27		1.47		0.05		0.96			2.13			4.37		0.61			0.05	1.41	
VS12	0.08			1.17		0.10		0.34			1.48					3.86			0.08	1.55	
VS13	0.17	0.20		0.70		0.05		0.70			0.50	0.07	2.53			0.51			0.07	3.12	
VS15		0.67		0.53				1.85			0.55					2.07			1.23	2.42	
VS17	0.08	1.02				0.07		0.40			0.51	0.15	3.37			0.39			0.09	3.06	
VS18	0.21			0.56				0.78			0.02	0.27	2.10			0.52			0.05	1.08	
VS19	0.13	0.25		0.76		0.07		0.81			1.03	0.12	2.40			0.60			0.08	2.81	
VS20	0.08	0.31						0.30			0.44		1.06			0.23			0.51	0.17	
VSV02	0.32	0.25		0.91				0.85			0.74		3.04			0.70				4.26	
VSV03	0.03	0.47		1.13		0.04		1.39			0.87		3.59			0.92			0.06	4.24	
VSV04		0.88						0.20			2.12	0.16	3.41							0.84	
VSV06	0.09	0.50						0.44			0.50	0.13	1.46			0.32			0.16	1.18	
VSV07		0.77						0.45			1.01		2.23							4.44	
VSV10		0.93				0.11		0.12			0.81	0.09	2.51			0.10			0.03	3.01	
VSV11	0.15	0.62						0.54			1.28		2.11			0.40			0.04	0.95	
VSV18	0.06	0.11				1.01		0.42			0.99		3.14			0.34			0.10	1.49	
VSV19				0.17		0.36		0.58			0.61	0.13	1.03			0.45				1.84	
VSV20	0.06	0.62				0.05		0.40			0.64	0.09	2.44			0.34			0.06	2.00	

Table 5-1. Percentage peak area matrix. Undetectable peaks are shaded.

(Continued)

R.T / Sample	28.73	28.78	28.90	28.98	29.04	29.15	29.27	29.36	29.37	29.49	29.52	29.53	29.71	29.85	29.88	29.91	29.92	30.24	30.26	30.27	30.34
AL1	2.10			2.00	1.47						2.14		0.16				0.58				0.83
AL11				5.61							2.39		0.30				0.07				1.28
AL12				7.13							2.53		0.18				0.08				0.49
AL15	0.99			4.76							2.86		0.25				0.08				1.32
AL16	0.96			3.46	2.11						3.28		0.21				0.09				1.16
AL17	2.94			3.93	2.16						2.13		1.47				0.61				1.39
AL21	1.28			4.33	2.48						2.06		1.86				0.43				1.54
AL22	1.17			2.64	2.25						2.74		0.27				0.16				1.15
AL23	1.22			5.39							2.99		0.21				0.21				1.15
AL25					7.80						2.62		2.66				0.28				1.40
AL25a				6.08							2.77		0.34				0.28				1.13
AL28	1.10			4.96	2.04						2.03		2.05				0.30				1.25
IH02				0.85				0.33									0.17				
IH07	1.35			5.00							1.97						2.42			0.25	
IH08	0.48			1.50				0.92			0.78									0.16	
IH11				1.91				2.21									0.71			0.16	
IH12	0.57			2.40				1.50									1.35			0.24	
IH13	2.98			2.51							1.88						1.26			0.40	
IH16	0.92			1.60				0.88									0.48			0.16	
IH17	0.46			1.43				1.03									0.92				
IH18	0.53			6.35				1.84									0.85			0.26	
IR01	0.41			0.43				0.34													
IR03	2.58			4.62				1.38			0.75						1.12				
IR05	1.25			4.62				2.06									0.83			0.12	
VB01			0.29			0.24				2.35	0.05										
VB03			1.47			1.87				2.06						0.33				0.08	
VB05			0.46			1.76				2.05						0.11				0.06	
VS02		5.14			2.83		2.53		1.91					0.49		1.63		0.14			
VS07	0.48	3.59			1.58		1.62		0.22			0.77		0.63		0.22		0.24	0.30		
VS08	1.96	6.77			2.04				2.86			0.79		0.77				0.28	0.31		
VS09	2.48	5.54			1.65		4.84		1.95			0.37		1.94					0.07		
VS10		4.54			2.14				3.32			1.08		0.72				0.26	0.50		
VS12		4.02			2.84				4.87			1.41		0.47		0.86		0.24			
VS13		4.14			2.00		2.74		0.95			0.62		0.47				0.08	1.40		
VS15	0.59	2.26			1.67		0.66		2.09			1.51		0.97		0.37			0.11		
VS17	5.75	3.22			5.89				1.93			0.52		1.83					0.11		
VS18		5.00					3.28		3.57			0.71		0.56		0.24					
VS19		4.64			2.08		3.03		1.59					0.50		1.41			0.13		
VS20	1.55	1.94			1.49				0.66					0.33					0.17		
VSV02	5.16	2.86			2.37		0.71		1.41							1.47		0.20			
VSV03	9.76	2.04			2.63		1.45		0.76					0.57		2.40		0.28	0.10		
VSV04	8.08	4.35			2.96				0.97							0.62		0.77	0.26		
VSV06	2.14	1.43			1.53		0.91		0.34							0.73		0.22	0.16		
VSV07	5.86	3.41			3.26		1.29		1.69							2.65		0.37			
VSV10	7.05	3.79			4.14		0.89		0.62					0.38		1.39		0.49	0.10		
VSV11	3.01	2.19			0.82				0.71					0.38		0.30		0.18			
VSV18	3.45				2.90		6.76		1.31					0.55		0.96		0.20			
VSV19	3.11	2.22			2.57		1.13		0.45							0.84		0.19	0.08		
VSV20	2.35				2.93		3.62		1.10					0.46		1.00					

R.T / Sample	30.50	30.53	30.61	30.63	30.69	30.70	31.03	31.14	31.14	31.15	31.20	31.48	31.64	31.65	31.83	31.87	32.11	32.24	32.24	32.45	32.87
AL1						0.85		0.28						0.13				0.37		1.07	
AL11						1.03		0.12						0.36	0.07			0.28		1.36	
AL12						0.79		0.44						0.30				0.22		0.52	
AL15						0.85		0.16						0.30	0.03			0.32		1.01	
AL16						1.23		0.07						0.24	0.04			0.44		1.16	
AL17						1.07		0.10						0.17	0.04			0.42		0.54	
AL21						0.93		0.06						0.11	0.04			0.63		0.95	
AL22						0.73		0.31						0.18	0.03			0.28		1.50	
AL23						0.65		0.06						0.15	0.03			0.61		0.81	
AL25						0.96		0.11						0.38	0.05			0.30		0.62	
AL25a						0.41		0.17						0.41	0.03			0.26		1.21	
AL28						0.57		0.21						0.31	0.04			0.25		0.94	
IH02		0.45		0.11				0.08													
IH07		0.22		0.51		0.35		0.09									0.07				
IH08				0.43				0.07									0.06			0.07	
IH11		0.15		0.61		0.07		0.11									0.04			0.05	
IH12		0.94		0.51																	
IH13		0.89		0.27		0.78		0.12									0.17			0.07	
IH16				0.43		0.44		0.07									0.07			0.10	
IH17				0.21																	
IH18				0.61				0.79												0.26	
IR01		0.99																			
IR03		0.62		0.86				0.93													
IR05		0.21		0.97				0.34													
VB01					0.61						0.08				0.23						
VB03					0.90						1.28				0.19				0.14		
VB05					0.79										0.30				0.13		
VS02	0.34		1.74			1.33					1.45	0.31	0.39		0.08	0.08		0.77	0.06		
VS07	0.35					1.69				1.23	1.90	0.40	0.36					1.91	0.08		0.09
VS08	0.32		1.01			0.71				1.50	3.41	0.68	0.45		0.06	0.12		0.81	0.09		0.15
VS09	0.32		1.55			1.34					3.91	0.80	0.44					1.60	0.04		0.10
VS10	0.39		1.24			0.71				1.58		3.87	0.77					1.25	0.15		0.18
VS12			1.26			0.78					7.10	1.07	0.71		0.10	0.16		1.42	0.06		0.26
VS13	0.25		0.17			1.31				1.30	1.92	0.43	0.35		0.05	0.05		0.77	0.05		
VS15	0.40		0.96			0.71				1.61	2.73	0.56	0.37		0.07	0.08		0.17	0.05		0.09
VS17	0.32		1.89			1.64					5.65	1.12	0.63		0.09			1.27	0.13		0.16
VS18	0.29		0.88			0.39				1.29		4.47	0.88		0.03	0.55		0.73	0.06		0.15
VS19	0.43					1.70				0.99	2.32	0.49	0.43			0.10		0.89	0.13		0.34
VS20	0.57		0.35			0.71				1.55	0.31	0.25						0.67			0.20
VSV02	1.68		0.67			1.46				1.48	0.24		0.32					2.51			
VSV03			1.80			1.70				0.09		0.03	0.19		0.03	0.08		0.62	0.07		
VSV04	1.30		1.74			0.86				2.92	0.57		0.70					1.90	0.15		
VSV06	0.46		0.67			1.35				1.25	0.24		0.26		0.07			2.87			
VSV07	2.41		0.92			0.76				1.04			0.38					2.71			
VSV10	0.82		0.33			0.12				5.37			0.90		0.11	0.61		1.39	0.05		0.19
VSV11	0.23		1.17			0.94					0.70	0.14	0.38		0.12	0.13		1.24			
VSV18	0.44		1.10			0.68				8.32	1.39	0.75			0.12	0.09		1.04	0.08		0.27
VSV19			0.87			0.67				2.28	0.47	0.38				0.06		1.02			
VSV20	0.47		0.93			0.30				1.61		4.02	0.78		0.13	0.61					2.85

Table 5-1. Percentage peak area matrix. Undetectable peaks are shaded.

(Continued)

Sample \ R.T	32.87	32.97	33.20	33.33	33.52	33.54	33.65	33.77	33.87	34.00	34.09	34.19	34.55	34.59	34.62	34.89	34.99	35.11	35.27	35.29	35.32
AL1			0.09			0.46			0.26		3.35	1.41			0.05		0.07		0.10		
AL11			0.10			0.42			0.21		2.05	2.02			0.28		0.21		0.15		
AL12			0.26			0.12			0.40		0.74	1.42			0.06		0.53		0.43		
AL15			0.09			0.41			0.23		1.60	1.26			0.18		0.16		0.14		
AL16			0.07			0.39			0.26		1.92	1.42			0.09		0.10		0.07		
AL17			0.06			0.39			0.30		2.45	1.96			0.05		0.08		0.05		
AL21			0.09			0.46			0.25		2.73	2.54			0.10		0.08		0.07		
AL22			0.12			0.27			0.22		2.61	2.35			0.06				0.16		
AL23			0.03			0.41			0.27		2.67	2.40			0.08				0.13		
AL25			0.06			0.34			0.27		1.06	0.96			0.04		0.10		0.10		
AL25a			0.11			0.36			0.19		1.07	0.72			0.22				0.13		
AL28			0.15			0.32			0.23		1.24	1.60			0.15		0.16				
IH02			0.06	0.04					0.77			0.25			0.06						
IH07			0.72	0.49			0.12		6.44			0.36			0.13		0.28				
IH08			0.08	0.07					1.99						0.10						
IH11			0.23				0.16		0.22	1.78			0.85		0.21						
IH12			0.28	0.17					2.74												
IH13			0.33	0.22			0.20		7.30			0.28			0.21		0.30				
IH16			0.27	0.13					3.28	0.43		0.19			0.14		0.16				
IH17			0.18						1.95	0.32		0.10									
IH18			0.15	0.19			0.12		3.88	0.63		0.24			0.24		0.18				
IR01								0.41													
IR03				0.61			0.23		6.40						0.18		0.15				
IR05			0.22	0.47			0.16		4.68			0.20			0.14		0.18				
VB01		0.43						1.45		0.06											0.14
VB03		0.78						0.65		0.45											0.53
VB05		0.93						1.11		0.35											0.47
VS02	0.29					0.13	0.08	0.06	0.50	0.73							0.21		0.80		
VS07	0.22						0.14	0.06		0.92	0.62				0.34		2.99		0.22		
VS08	0.37					0.07	0.19	0.05		0.54	0.63				0.36		1.14				
VS09	0.04							0.04		0.92	0.76				0.06		0.87				
VS10	0.16					0.20	0.20			0.80	1.06				0.26		3.56				
VS12	0.15					0.20	0.09			0.86					0.37		2.21				
VS13	0.27					0.10				0.60	0.59				0.18		0.48				
VS15	0.08					0.08	0.05	0.07		0.47					0.51		1.19				
VS17	0.33					0.08	0.14	0.07		0.59	1.11				0.26		1.32				
VS18	0.12					0.35	0.05			0.46	0.50				0.20		0.62				
VS19						0.16	0.09	0.07		0.97	0.56				0.25		1.29				
VS20						0.14				0.20	0.40	0.19			0.55		1.25				
VSV02	0.27									0.72	0.91	0.37			2.46		0.23		0.56		
VSV03	0.19					0.07	0.09	0.04		0.64	0.72	0.23			1.26		0.24				
VSV04	0.13						0.18			0.83	0.82	0.49			1.99		0.19		1.26		
VSV06	0.29						0.14			1.03	0.87	0.35			3.78		0.29		0.92		
VSV07										0.52	0.87	0.65			2.72				0.34		
VSV10	0.09						0.11	0.07		0.41	0.90	0.15			3.95				2.69		
VSV11	0.27					0.29		0.07		0.33	0.53	0.44			1.57				0.61		
VSV18	0.19					0.11	0.11				0.42	0.21			0.09		0.72				
VSV19	0.26							0.07		0.49	5.87				6.23		1.14		1.26		
VSV20	0.55					0.13	0.11			0.53	1.11				0.31		1.95				

Sample \ R.T	35.50	35.64	35.67	35.72	35.96	35.99	36.08	36.18	36.18	36.67	37.06	37.07	37.14	37.37	37.41	37.49	37.63	37.87	37.91	37.93	38.04
AL1				0.12					0.58	0.21		0.93							0.08		
AL11				0.22					0.58			1.66					0.08		3.44		
AL12				0.19					0.42	0.10		0.72							8.14		
AL15				0.10					0.50	0.18		1.14					1.73		0.48		
AL16									0.50	0.04		0.65							1.85		
AL17				0.15					0.31	0.05		0.28							0.20		
AL21				0.18					0.31	0.59		0.27							1.10		
AL22				0.15					0.81	0.45		2.11					1.50		2.66		
AL23				0.16					0.48	0.05		1.05							2.39		
AL25				0.02					0.38	0.09		0.29							0.39		
AL25a				0.59					0.16			1.64					0.06				
AL28				0.15					0.39	0.11		0.57					0.41		0.99		
IH02													0.13	0.18							
IH07												0.57			0.55		0.27				
IH08												0.15	0.68				0.10				
IH11												0.29	0.67				0.14		0.26		
IH12												0.14	0.61								
IH13												0.34		2.13					0.33		
IH16												0.37		0.96			0.19		0.19		
IH17												0.22		0.47							
IH18						0.51						0.50		1.42					0.36		
IR01																					
IR03												0.34									
IR05												0.41	0.92								
VB01																					
VB03																			0.37		
VB05																			0.95		
VS02		0.61					1.22	0.58		0.37	1.84					0.82					3.85
VS07	0.81		0.38				0.61	1.27		0.47	1.18					0.42		0.76			2.49
VS08	1.47				0.51		0.82	1.37		0.20	0.75					0.97					2.33
VS09	2.07	0.79					0.96	1.28		0.24	1.51							1.51			4.19
VS10	1.75				0.56		0.91	1.57		0.47	1.58					1.14					3.56
VS12	2.77				0.72		1.30	1.27		0.30	1.85							1.93			
VS13	1.19				0.55		0.74	0.86		0.20	1.37							0.92			3.74
VS15	0.27				0.16		0.70	0.70		0.24	0.66							0.83			2.46
VS17	2.59						1.38			0.24	1.31							1.68			4.63
VS18	2.68		0.46							0.13	1.85										
VS19	1.07		0.58				1.53			0.28	1.20										
VS20	0.57						0.29	0.64		0.15	1.52										
VSV02		0.59					0.99	0.65		0.51	1.48					0.52		2.73			1.71
VSV03		0.53					1.53			0.26	0.84					0.50					0.74
VSV04			0.49		0.47		0.70	1.30		0.23	0.30	1.23				1.16		2.80			1.32
VSV06			0.56		0.72		0.90	0.96		0.53	0.75	1.80				0.96		3.54			4.37
VSV07			0.76				2.45			0.25	0.89					0.43		1.63			1.58
VSV10					0.67			1.24		0.92		0.58				2.64		2.12			
VSV11											2.70										
VSV18							1.25	1.18		0.41	2.73							2.22			
VSV19		0.37			0.54		0.26	0.39		0.18	0.42	0.95				0.99					6.35
VSV20								1.20		1.24	2.24	0.38						1.78			5.64

Table 5-1. Percentage peak area matrix. Undetectable peaks are shaded.

(Continued)

R.T / Sample	38.10	38.12	38.20	38.26	38.32	38.62	38.62	38.76	39.03	39.09	39.17	39.60	39.64	39.73	39.85	39.86	39.91	39.97	39.98	39.98	40.05
AL1											34.76										0.64
AL11			3.52								26.43										0.70
AL12											32.16										15.35
AL15			0.60								25.79										8.85
AL16			2.84								34.09										0.65
AL17			1.32								34.94										0.65
AL21			0.90								32.42										1.24
AL22											29.33										0.73
AL23											35.43										0.54
AL25			1.05								38.39										0.86
AL25a			3.73								28.70										0.61
AL28			1.07								35.37										1.21
IH02		0.31		0.26			0.21	0.46	0.83								0.17		0.12		
IH07				1.17			0.61	3.40	2.70				0.61				0.30				
IH08	0.08	0.68		0.47			0.40	0.77	0.55		0.30		0.26				0.26				
IH11	0.62	0.45					2.17	16.51									0.40		0.28		
IH12		0.76		0.28			0.77	1.06	0.47				0.20				0.24				
IH13		0.89		0.93			1.48	1.92	4.67		0.81						0.81				
IH16		0.71		0.59			0.88	1.20	3.51				0.41				0.41				
IH17		0.54		0.25			0.38	0.74	3.60				0.13				0.18				
IH18		2.11		0.68			0.83	1.36	1.21		0.54						0.73		0.72		
IR01		0.34		0.20			0.33	0.45	1.87				0.08								
IR03				1.46			1.97	2.56	1.47		0.67		0.61				0.36				
IR05				1.31			0.39	4.16	6.84								0.81		0.65		
VB01																	80.03				
VB03																	60.69				
VB05																	66.60				
VS02					1.29	1.71		3.19				10.35									
VS07					0.67	3.09		2.08		1.28		6.83		0.30							
VS08					0.52	1.15				2.76		7.62		0.23							
VS09						4.43						12.84		0.22							
VS10						3.37		3.21		4.84		0.32		0.18							
VS12					6.39	2.02				3.93		7.34		0.32	0.30						
VS13					0.68	0.45		0.75		2.68		0.98		7.24	0.32			1.21			
VS15					0.52	0.36		0.77		2.14		8.14		0.23							
VS17					1.02	1.27		2.64				10.54		1.62							
VS18					13.43	0.88				3.24		6.34		0.44							
VS19					7.74	1.72				4.27		9.90									
VS20					26.62	1.01		2.42		9.28		1.79									
VSV02					2.23	1.15		1.52		11.58				1.98							
VSV03					1.84			2.84		6.03		0.23		2.45					0.31		
VSV04								1.32		12.78		0.18		1.06							
VSV06										9.78				5.45							
VSV07					2.29	1.93		1.02		6.93				1.27							
VSV10					5.83	0.76		3.95		3.09		8.04		0.40	0.28			0.14			
VSV11					14.43			1.70		5.08		8.40									
VSV18					8.30	1.47		2.44		2.54		7.72		0.38	0.27			0.48		0.85	
VSV19								3.67		13.65							2.73		0.44		
VSV20					1.29	1.86		3.75		6.32		0.52		0.27							

R.T / Sample	40.11	40.16	40.17	40.19	40.28	40.37	40.48	40.77	40.81	40.81	40.89	40.99	41.04	41.13	41.28	41.36	41.43	41.46	41.52	41.67	41.70
AL1				0.74		0.80		0.90			0.33		0.10				0.44		0.60	0.28	0.11
AL11				0.84		0.51		1.22			0.15						0.80		1.36	0.48	0.22
AL12				0.61		0.40		0.31			0.57		0.53				0.42		0.27	0.18	0.41
AL15				0.74		0.56		1.16			0.42		0.09				0.58		0.96	0.39	0.15
AL16				0.69		0.30		0.93			0.32						0.55		0.93	0.34	0.17
AL17				0.52		0.32		0.87			0.29		0.39				0.51			0.25	0.10
AL21				0.38		0.63					0.43		0.50				0.19		0.14	0.30	0.11
AL22				0.83		1.02		0.51			0.85		0.48				0.44		0.10	0.31	
AL23				0.93		0.47		0.14			0.74		0.65				0.21		0.18	0.44	0.17
AL25				0.50		0.46		0.96			0.34		0.35				0.61		0.06	0.18	
AL25a				0.63		0.39		0.76			0.52		0.53				0.39		0.13		0.32
AL28				0.37		0.79					0.47		0.06				0.38		0.46	0.25	0.10
IH02								1.48					0.33		0.18				0.30		
IH07								0.60					0.38		0.17				0.27		
IH08													0.35						0.32	0.39	
IH11								0.56					0.50		0.15				0.22	0.41	
IH12								3.08					0.20								
IH13								2.63					0.87		0.22				0.44	0.79	
IH16													0.55						0.46	0.57	
IH17													0.14								
IH18								0.14					0.56						0.27	0.47	
IR01								2.70						0.39							
IR03								1.49					0.28		0.20				0.19		
IR05								0.61					0.53		0.27				0.22	0.39	
VB01							0.94	0.57		0.95											
VB03							1.06	0.65		0.86								0.20			
VB05							0.86	0.43		0.71								0.54			
VS02			2.99		0.66			0.38	0.68		0.22	0.15				0.54					
VS07			3.82		0.76				0.79				0.10			0.49			0.18		
VS08			3.01		0.76				0.45			0.06	0.13			0.41					
VS09			1.44		0.84			0.16	0.38			0.45	0.14			0.75			0.70		
VS10			2.59		1.13			0.14	0.60				0.08			0.65			0.09		
VS12			1.25						1.46			0.48	0.17			0.78					
VS13		0.33	2.68					0.34	0.81			0.25	0.19			0.50			0.49		
VS15		0.32			4.53				0.23			0.08	0.10			0.17			0.17		
VS17					0.76				1.64			0.38				0.51					
VS18					4.58			0.25	0.64			0.15	0.11			0.39			0.13		
VS19					2.80			0.91	0.78			0.33	0.17			0.41					
VS20					0.51			1.34	0.28			0.22									
VSV02	0.42							4.30	0.31												
VSV03	0.23	0.11	1.11		0.25			0.31				0.41				0.56					
VSV04	1.13							2.87	0.20							0.58					
VSV06	1.20							0.87	0.20			0.24				0.60			0.35		
VSV07	0.88		0.53					7.83	0.90												
VSV10	0.93		0.50		0.12			0.28	0.46			0.27				0.77					
VSV11			0.77		1.95			1.55	0.90			0.25				0.83			0.37		
VSV18	1.12	0.99						0.12	0.19			0.39				0.11					
VSV19	0.80											0.20	0.12			0.31			0.23		
VSV20			2.81		1.11				0.69			0.32	0.24			0.69					

Table 5-1. Percentage peak area matrix. Undetectable peaks are shaded.

(Continued)

R.T / Sample	41.73	41.90	42.16	42.54	42.80	42.84	42.99	43.05	43.16	43.26	43.44	43.60	43.70	43.81	43.93	44.19	44.33	44.42	44.56	44.84	45.05
AL1						0.04		1.56	0.04				0.21	1.98			2.85		1.99		
AL11						0.43		1.98					0.31	0.11			3.70		3.27		
AL12						0.03		0.39					0.03	0.01			0.99		0.37		
AL15						0.22		1.66					0.34	0.08			5.08		2.72		
AL16						0.13		0.99	0.07				0.19	0.08			3.38		2.00		
AL17						0.06		0.03	0.75				0.12				3.14		1.33		
AL21						0.07		0.69					0.16	2.28			1.57				
AL22						0.13		1.28					0.32	2.93			2.45				
AL23						0.07		1.01					0.26	2.95			1.96				
AL25						0.03		1.04					0.07	3.33			1.29				
AL25a						0.19		1.76					0.11	0.08			3.53		1.81		
AL28						0.05		1.07					0.17	3.12			1.65				
IH02												0.10									
IH07								0.23						0.11							
IH08								0.11													
IH11								0.11	0.14												
IH12									0.19												
IH13								0.47				0.12									
IH16								0.58				0.14					0.24				
IH17																					
IH18								0.57	0.11			0.16					0.13				0.07
IR01												0.18									
IR03								0.49						0.09							
IR05								0.81				0.16		0.06			0.23		0.05		
VB01		0.47																			
VB03		0.53																			
VB05		0.93						0.08													
VS02	0.53		0.24	0.15	0.10		0.26	1.42			0.17						1.75	0.82		0.16	0.74
VS07	0.21		0.24	0.11	0.09		0.19	1.26			0.13				0.16		1.15	0.57		0.06	0.85
VS08	0.14		0.18		0.06		0.19	0.90			0.06						1.03	0.31		0.04	0.71
VS09								1.63			0.19				0.06		1.95	1.11		0.17	0.32
VS10	0.17		0.12	0.08			0.20	1.24			0.11				0.08		1.34	0.58		0.09	0.63
VS12	0.38		0.11	0.13			0.06	1.28			0.10				0.08		1.51	0.78		0.14	0.28
VS13	0.17		0.22	0.17			0.08	0.24		1.75	0.23				0.19	0.70	1.03	0.93		0.20	0.91
VS15	0.20		0.12	0.04	0.07		0.41			1.26					0.07	0.74	0.67	0.44			
VS17	0.42		0.06	0.05				1.10			0.11				0.92			0.35		0.08	0.34
VS18	0.22		0.16	0.68	0.16		0.24			1.40	0.10				0.06	1.49	1.15	0.73			1.19
VS19	0.66		0.13	0.18			0.06	0.16		0.97	0.17				0.11	0.77	1.02	0.57		0.08	0.60
VS20	0.33		0.41		0.14		0.17	0.92										6.98			0.43
VSV02								0.67							0.68						0.55
VSV03	0.60		0.15		0.05		0.23	1.10			0.19				0.55		0.41	0.50		0.19	0.87
VSV04								0.97							1.75			0.73			
VSV06	0.28		0.23		0.10		0.35	2.10							1.66			0.81			1.32
VSV07								0.54							0.96						0.36
VSV10	0.45		0.19	0.11	0.07			1.31			0.15				0.14		1.33	0.77		0.29	0.25
VSV11	0.15		0.15	0.61				0.35			1.16				0.22		1.30	1.96		0.64	0.54
VSV18	0.22		0.15	0.23	0.07		0.08	1.17			0.10				0.11		1.00	1.06		0.93	0.10
VSV19	0.17		0.09	0.11	0.06		0.19	0.87									1.91	0.56			0.65
VSV20	0.68		0.23	0.20	0.38			1.60			0.23				0.19	0.74	1.19	0.91		0.15	0.88

R.T / Sample	45.42	45.55	45.82	45.89	46.00	46.03	46.50	46.51	46.66	46.72	46.98	47.11	47.19	47.33	47.43	47.71	47.80	47.89	47.93	48.06	48.53
AL1				0.45		0.10				0.03					0.52			0.04			0.05
AL11				0.33		0.18				0.04					0.44						0.09
AL12				0.18		0.08				0.15	0.09				0.21			0.12			0.03
AL15				0.46		0.18				0.04					0.62						0.07
AL16				0.33		0.10				0.04	0.02				0.68						0.04
AL17				0.38		0.11				0.03					0.35			0.01			0.02
AL21				0.28		0.05				0.03					0.31			0.01			0.02
AL22				0.31		0.13									0.55			0.03			0.06
AL23				0.47		0.08				0.03					0.50			0.03			0.03
AL25				0.42		0.16				0.04					0.31						0.05
AL25a				0.65		0.21				0.04	0.03				0.25			0.04			0.05
AL28				0.66		0.17									0.33						0.03
IH02						0.07		0.12					0.25		0.60		0.52				
IH07						0.06							0.39				0.76				
IH08													0.55				1.00				
IH11						0.09		0.07			0.06		0.31		0.60		0.61				
IH12													0.61				1.08				
IH13						0.13							0.96				1.83				
IH16													0.54				1.66				
IH17													0.53				0.98				
IH18													0.39				0.79				
IR01						0.13							0.27				0.55				
IR03						0.11		0.23					0.40		0.60		0.88				
IR05						0.05							0.37				0.80				
VB01										0.22	0.18										
VB03										0.74	0.82					0.37		0.14	0.43	0.52	
VB05										0.41	0.34				0.99			0.53	0.77	1.01	
VS02	0.11	0.08	0.38				0.54					0.06		0.16	0.48			0.26	0.46	0.64	
VS07		0.16	0.15		0.05		0.26		0.07			0.09							0.02		0.14
VS08	0.07		0.04				0.24		0.05			0.06		0.08					0.03		0.07
VS09	0.10		0.48		0.06		0.44							0.44					0.03		0.08
VS10		0.11	0.10				0.36		0.16			0.08		0.27					0.03		0.10
VS12	0.06	0.06	0.21				0.55					0.10		0.20					0.02		0.10
VS13	0.12	0.13	0.51				0.08		0.67			0.05		0.16					0.04		0.11
VS15	1.20	0.14	0.53				0.11					0.06		0.22					0.01		0.15
VS17	0.06		0.21		0.10		0.51														0.17
VS18	0.12		0.20		0.07		0.40					0.04		0.32					0.02		0.05
VS19	0.09	0.09	0.26		0.09		0.25		0.08			0.08		0.25					0.04		0.17
VS20			0.19		0.12		0.44												0.03		0.11
VSV02					0.20		0.28														
VSV03	0.08	0.11	0.23		0.05		0.35							0.13					0.01		0.05
VSV04					0.13		0.32							0.36							
VSV06		0.23	0.31				0.27		0.10			0.11							0.03		0.09
VSV07			0.37																		
VSV10	0.13		0.39		0.07		0.33		0.09			0.09		0.32					0.04		0.09
VSV11	0.05	0.17	0.51				0.41		0.20			0.16		0.29					0.06		0.17
VSV18	0.26	0.04			0.08		0.39		0.06			0.06		0.16					0.03		0.09
VSV19	0.05	0.06					0.15		0.05					0.31							0.07
VSV20	0.17	0.09	0.50		0.10							0.13		0.19					0.06		0.12

Table 5-1. Percentage peak area matrix. Undetectable peaks are shaded.

(Continued)

Sample \ R.T	48.81	49.06	49.43	49.45	49.68	50.12	50.28	50.44	50.50	50.54	50.83	50.83	50.88	51.01	51.42	51.55	51.84	51.84	52.02	52.33	52.45	52.65	52.78
AL1	0.00	0.05	0.00	0.11	0.06	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.14	0.00	0.00	0.05	0.04	0.00	0.06	0.00	0.00	0.00	2.94
AL11	0.00	0.00	0.00	0.27	0.13	0.00	0.00	0.00	0.00	0.00	0.45	0.00	0.14	0.00	0.00	0.00	0.03	0.18	0.00	0.00	0.00	0.00	1.22
AL12	0.00	0.03	0.00	0.56	0.06	0.00	0.07	0.00	0.00	0.00	0.96	0.00	0.00	0.06	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	1.87
AL15	0.00	0.10	0.00	0.28	0.10	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.05	0.00	0.13	0.10	0.00	0.11	0.00	0.00	0.00	1.97
AL16	0.00	0.05	0.00	0.18	0.07	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.05	0.00	0.00	0.08	0.04	0.00	0.00	0.00	0.00	0.00	1.18
AL17	0.00	0.00	0.00	0.06	0.03	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.77
AL21	0.00	0.00	0.00	0.08	0.04	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.05	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	2.86
AL22	0.00	0.08	0.00	0.23	0.08	0.00	0.02	0.00	0.00	0.00	0.12	0.00	0.00	0.05	0.00	0.04	0.09	0.00	0.00	0.00	0.00	0.00	3.10
AL23	0.00	0.04	0.00	0.13	0.06	0.00	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.00	0.00	0.08	0.07	0.00	0.00	0.00	0.00	0.00	3.49
AL25	0.00	0.04	0.00	0.16	0.07	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.03	0.00	0.11	0.05	0.00	0.09	0.00	0.00	0.00	1.49
AL25a	0.00	0.14	0.00	0.98	0.05	0.00	0.00	0.00	0.00	0.00	1.94	0.00	0.00	0.00	0.00	0.25	0.00	0.00	1.87	0.00	0.00	0.00	3.81
AL28	0.00	0.00	0.00	0.15	0.10	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.00	0.05	0.00	0.08	0.05	0.00	0.31	0.00	0.00	0.00	3.60
IH02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	48.42	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00
IH07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	21.12	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00
IH08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	45.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IH11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	33.82	0.08	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00
IH12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	36.76	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IH13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	17.08	0.15	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00
IH16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	31.16	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00
IH17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	46.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IH18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	23.26	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00
IR01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	52.21	0.19	0.00	0.00	0.00	0.26	0.00	0.00	0.00	0.00	0.00
IR03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	18.68	0.08	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00
IR05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	21.42	0.00	0.00	0.00	0.00	0.07	0.00	0.08	0.09	0.00	0.00
VB01	0.38	0.07	0.50	0.00	0.00	0.00	0.05	0.00	0.12	0.00	0.00	1.13	0.00	0.34	0.00	0.00	0.00	0.00	0.06	0.06	0.05	0.22	0.00
VB03	0.51	0.21	0.57	0.00	0.00	0.00	0.05	0.00	0.21	0.00	0.00	1.87	0.00	0.62	0.00	0.00	0.00	0.00	0.07	0.15	0.57	0.00	0.00
VB05	0.30	0.13	0.40	0.00	0.00	0.00	0.04	0.00	0.98	0.00	0.00	2.08	0.00	0.60	0.00	0.00	0.00	0.00	0.04	0.47	0.42	0.00	0.00
VS02	0.00	0.06	0.39	0.00	0.17	0.11	0.00	0.00	0.00	0.07	0.27	0.00	0.00	0.03	0.00	0.03	0.03	0.00	0.18	0.00	0.00	0.00	5.45
VS07	0.00	0.04	0.50	0.00	0.05	0.04	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.06	0.00	0.04	0.08	0.00	0.04	0.11	0.00	0.00	1.37
VS08	0.00	0.02	0.33	0.00	0.04	0.00	0.00	0.00	0.05	0.05	0.00	0.00	0.00	0.04	0.00	0.00	0.02	0.00	0.04	0.00	0.00	0.00	1.05
VS09	0.00	0.00	0.67	0.00	0.10	0.18	0.00	0.00	0.00	0.04	0.18	0.00	0.00	0.04	0.00	0.04	0.05	0.00	0.10	0.00	0.00	0.00	2.87
VS10	0.00	0.00	0.36	0.00	0.07	0.06	0.00	0.00	0.00	0.03	0.04	0.00	0.00	0.03	0.00	0.02	0.03	0.00	0.03	0.09	0.00	0.00	0.72
VS12	0.00	0.05	0.60	0.00	0.13	0.07	0.00	0.00	0.00	0.06	0.19	0.00	0.00	0.00	0.00	0.03	0.02	0.00	0.10	0.00	0.00	0.00	2.37
VS13	0.00	0.15	0.40	0.00	0.18	0.17	0.00	0.00	0.00	0.11	0.31	0.00	0.00	0.06	0.00	0.05	0.10	0.00	0.21	0.00	0.00	0.00	6.59
VS15	0.00	0.00	0.00	0.00	1.35	0.03	0.00	0.00	0.00	0.06	0.95	0.00	0.00	0.00	0.00	0.05	0.06	0.00	0.66	0.99	0.00	0.00	2.44
VS17	0.00	0.00	0.45	0.00	0.05	0.10	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00
VS18	0.00	0.05	0.67	0.00	0.14	0.03	0.00	0.00	0.10	0.15	0.00	0.00	0.00	0.05	0.00	0.06	0.06	0.00	0.25	0.00	0.00	0.00	2.37
VS19	0.00	0.07	0.77	0.00	0.07	0.12	0.00	0.00	0.00	0.11	0.11	0.00	0.00	0.07	0.00	0.04	0.06	0.00	0.05	0.00	0.00	0.00	2.18
VS20	0.00	0.00	0.27	0.00	0.08	0.00	0.00	0.00	0.42	0.33	0.00	0.00	0.00	0.00	0.00	0.10	0.12	0.00	0.19	0.00	0.00	0.00	7.76
VSV02	0.00	0.00	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	0.00	0.41	0.00	0.00	0.00	0.00	0.57
VSV03	0.00	0.02	0.21	0.00	0.04	0.10	0.00	0.00	0.00	0.04	0.04	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.09	0.00	0.00	0.00	1.01
VSV04	0.00	0.00	0.42	0.00	0.00	0.00	0.70	0.00	0.00	0.16	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	4.10
VSV06	0.00	0.07	0.47	0.00	0.09	0.00	0.00	1.32	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.10	0.00	0.00	0.00	5.15
VSV07	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.00	0.00	0.00	0.00	0.00	0.69	0.00	0.00	2.95
VSV10	0.00	0.07	0.46	0.00	0.11	0.11	0.00	0.00	0.43	0.17	0.00	0.00	0.00	0.07	0.00	0.06	0.07	0.00	0.14	0.14	0.00	0.00	2.26
VSV11	0.00	0.35	0.69	0.00	0.18	0.08	0.00	0.00	0.00	0.14	0.65	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.44	0.00	0.00	0.00	6.12
VSV18	0.00	0.04	0.53	0.00	0.13	0.05	0.00	0.03	0.00	0.03	0.16	0.00	0.00	0.06	0.00	0.04	0.06	0.00	0.12	0.00	0.00	0.00	2.16
VSV19	0.00	0.00	0.52	0.00	0.10	0.06	0.00	0.12	0.00	0.00	0.24	0.00	0.00	0.00	0.00	0.06	0.04	0.00	0.19	0.00	0.00	0.00	4.23
VSV20	0.00	0.09	0.63	0.00	0.12	0.22	0.00	0.00	0.00	0.30	0.25	0.00	0.00	0.00	0.00	0.04	0.07	0.00	0.17	0.00	0.00	0.00	5.73

Sample \ R.T	52.82	52.83	52.95	53.16	53.44	53.45	53.54	53.61	53.83	53.87	54.90	54.92	55.18	55.53	55.55	55.60	55.75	55.77	56.85	56.87	57.99	60.34
AL1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.51	0.03	0.00	0.00	0.00	0.00	0.06
AL11	0.00	1.96	0.00	0.00	0.00	0.00	0.00	0.44	0.00	0.00	0.00	0.06	0.00	0.00	0.00	9.15	0.00	0.00	0.00	0.00	0.00	0.03
AL12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.03	0.00	0.00	0.00	4.84	0.00	0.00	0.00	0.02	0.00	0.07
AL15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.46	0.02	0.00	0.00	0.04	0.00	0.00	0.00	10.85	0.07	0.00	0.00	0.07	0.00	0.07
AL16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.03	0.00	0.00	0.00	0.00	0.00	0.00	8.81	0.03	0.00	0.00	0.00	0.00	0.05
AL17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.00	3.71	0.02	0.00	0.00	0.00	0.00	0.03
AL21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.03	0.00	0.00	0.00	4.12	0.00	0.00	0.00	0.00	0.00	0.05
AL22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00	0.00	0.04	0.00	0.00	0.00	10.74	0.06	0.00	0.01	0.00	0.00	0.06
AL23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.03	0.00	0.00	0.00	8.07	0.00	0.00	0.00	0.00	0.00	0.00
AL25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.00	0.03	0.00	0.00	0.00	5.74	0.07	0.00	0.00	0.02	0.00	0.05
AL25a	0.00	0.00	0.00	0.00	0.00	0.00	4.59	0.08	1.11	0.00	0.10	0.00	0.00	0.00	0.00	6.73	0.04	0.00	0.00	0.22	0.00	0.07
AL28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.46	0.03	0.00	0.00	0.05	0.00	0.06
IH02	0.00	35.47	0.00	0.24	0.08	1.24	0.00	0.00	0.00	0.00	0.04	1.24	0.00	0.09	0.00	0.00	0.00	0.21	0.00	0.08	0.05	0.27
IH07	0.00	16.54	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	1.14	0.00	1.25	0.00	0.00	0.00	0.34	0.00	0.00	0.00	0.08
IH08	0.00	26.34	0.00	0.10	0.00	0.90	0.00	0.00	0.00	0.00	0.00	0.81	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.39
IH11	0.00	15.57	0.00	0.10	0.04	0.61	0.00	0.00	0.00	0.00	0.00	0.42	0.00	0.08	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.13
IH12	0.00	21.64	0.24	0.69	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.74	0.00	0.28	0.00	0.00	0.00	0.14	0.00	0.27	0.00	0.58
IH13	0.00	18.03	0.10	0.15	0.10	1.80	0.00	0.00	0.00	0.00	0.00	0.24	1.78	0.00	0.33	0.00	0.00	0.18	0.00	0.13	0.13	0.47
IH16	0.00	28.25	0.00	0.18	0.00	2.74	0.00	0.00	0.00	0.00	0.00	0.18	3.37	0.00	0.09	0.00	0.00	0.12	0.00	0.00	0.00	0.00
IH17	0.00	26.16	0.24	0.00	0.00	0.87	0.00	0.00	0.00	0.00	0.00	0.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
IH18	0.00	19.26	0.00	0.12	0.00	1.47	0.00	0.00	0.00	0.00	0.30	1.32	0.00	0.06	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00
IR01	0.00	30.12	2.21	0.20	0.00	0.71	0.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.44	0.00	0.00	0.39	0.42	
IR03	0.00	15.54	0.10	0.11	0.08	1.18	0.00	0.00	0.00	0.00	0.00	1.29	0.00	0.00	0.12	0.00	0.00	0.00	0.07	0.14	0.40	
IR05	0.00	19.74	0.00	0.15	0.06	1.23	0.00	0.00	0.00	0.00	0.00	1.09	0.00	0.17	0.00	0.00	0.12	0.00	0.03	0.12	0.09	
VB01	0.24	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.08
VB03	0.52	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.10	0.19	0.12	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.09
VB05	0.41	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.04	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06
VS02	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	6.38	0.03	0.00	0.12	0.00	0.00	0.08
VS07	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.02	0.00	0.00	0.04	0.00	0.00	5.21	0.04	0.00	0.05	0.00	0.00	0.07
VS08	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.01	0.00	0.00	0.02	0.00	0.00	3.18	0.02	0.00	0.02	0.00	0.00	0.05
VS09	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	8.16	0.02	0.00	0.18	0.00	0.00	0.07
VS10	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.02	0.00	0.00	0.00	0.02	0.00	0.00	2.65	0.02	0.00	0.01	0.00	0.00	0.04
VS12	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	5.04	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.03
VS13	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.12	0.00	0.02	0.00	0.00	8.29	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.08
VS15	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.24	0.00	0.00	0.00	0.00	2.22	0.00	0.00	0.02	0.00	0.06	0.00	0.00	0.00	0.03
VS17	0.00	0.00	0.00	0.00	0.00	1.69	0.00	0.07	0.00	0.05	0.00	0.00	0.15	0.00	0.00	3.89	0.13	0.00	0.09	0.00	0.00	0.16
VS18	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.03	0.00	0.00	0.00	0.00	5.10	0.00	0.00	0.00	0.03	0.00	0.03	0.00	0.00	0.08
VS19	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.02	0.00	0.00	0.04	0.00	0.00	4.22	0.04	0.00	0.08	0.00	0.00	0.06
VS20	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.13	0.00	0.00	0.00	0.08	0.00	0.00	0.00	9.28	0.09	0.00	0.40	0.00	0.00	0.25
VSV02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	1.28	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.21	0.00	0.00	0.00	0.50
VSV03	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.02	0.00	3.63	0.00	0.00	0.02	0.00	0.00	0.02	0.00	0.04	0.00	0.00	0.00	0.03
VSV04	0.00	0.00	0.00	0.00	0.00	0.18	0.00	0.13	0.00	9.66	0.00	0.00	0.16	0.00	0.00	0.25	0.00	0.18	0.00	0.00	0.00	0.44
VSV06	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.06	0.00	12.99	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.10	0.00	0.00	0.00	0.14
VSV07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	6.07	0.00	0.00	0.00	0.00	0.00	0.65	0.00	0.41	0.00	0.00	0.00	1.22
VSV10	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.03	0.00	6.11	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.02
VSV11	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.25	0.00	8.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.07
VSV18	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.15	0.00	0.03	0.00	0.00	0.00	0.05	0.00	4.46	0.05	0.00	0.05	0.00	0.00	0.11
VSV19	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.03	0.00	0.00	0.00	0.00	0.00	5.79	0.00	0.00	0.05	0.00	0.13	0.00	0.00	0.07
VSV20	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.07	0.00	0.03	0.00	0.00	0.00	6.90	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.05

Table 5-2. Pairwise relationship between *Aucklandia lappa* and related samples. Upper diagonal: Dissimilarity matrix showing the pairwise distance calculated from squared Euclidean Distance. Lower diagonal: Similarity matrix showing the pairwise similarity calculated from Cosine of vectors of variables.

Sample	1:AL1	2:AL11	3:AL12	4:AL15	5:AL16	6:AL17	7:AL21	8:AL22	9:AL23	10:AL25	11:AL25a	12:AL28	13:IH02	14:IH07	15:IH08	16:IH11
1:AL1		166	383	215	35	58	71	81	52	131	145	71	4985	2154	4045	3017
2:AL11	0.950		343	108	99	216	150	80	142	315	79	176	4366	1743	3512	2509
3:AL12	0.866	0.877		224	332	385	327	348	311	462	365	313	5039	2325	4140	3094
4:AL15	0.927	0.943	0.926		172	282	238	137	227	399	146	261	4563	1867	3674	2628
5:AL16	0.988	0.974	0.882	0.939		61	81	78	58	131	98	77	4940	2167	4019	2984
6:AL17	0.980	0.928	0.866	0.898	0.978		68	174	108	154	164	81	4987	2115	4037	3006
7:AL21	0.976	0.940	0.881	0.898	0.970	0.977		90	52	115	138	24	4808	1986	3872	2838
8:AL22	0.976	0.964	0.872	0.935	0.974	0.939	0.963		64	178	120	102	4686	1965	3783	2744
9:AL23	0.982	0.964	0.893	0.925	0.980	0.962	0.984	0.985		133	134	32	5038	2272	4120	3084
10:AL25	0.960	0.915	0.853	0.878	0.961	0.953	0.971	0.955	0.959		279	81	5253	2582	4357	3323
11:AL25a	0.953	0.961	0.865	0.927	0.969	0.945	0.943	0.944	0.960	0.922		136	4615	1902	3720	2676
12:AL28	0.975	0.944	0.891	0.905	0.972	0.971	0.993	0.967	0.989	0.977	0.956		4992	2236	4075	3038
13:IH02	0.011	0.048	0.005	0.006	0.008	0.010	0.009	0.005	0.006	0.003	0.006	0.006		1373	126	899
14:IH07	0.132	0.124	0.073	0.080	0.105	0.147	0.133	0.087	0.096	0.049	0.088	0.093	0.844		821	496
15:IH08	0.038	0.060	0.021	0.022	0.030	0.039	0.036	0.024	0.026	0.017	0.024	0.026	0.989	0.879		503
16:IH11	0.037	0.053	0.022	0.021	0.029	0.040	0.037	0.023	0.025	0.013	0.024	0.025	0.890	0.845	0.914	
17:IH12	0.071	0.074	0.033	0.035	0.053	0.076	0.068	0.041	0.044	0.026	0.039	0.045	0.957	0.938	0.980	0.908
18:IH13	0.131	0.139	0.077	0.089	0.107	0.138	0.126	0.098	0.102	0.065	0.088	0.093	0.871	0.953	0.879	0.814
19:IH16	0.039	0.071	0.020	0.022	0.029	0.040	0.037	0.025	0.027	0.013	0.023	0.025	0.980	0.898	0.971	0.877
20:IH17	0.032	0.052	0.015	0.015	0.023	0.034	0.030	0.018	0.020	0.010	0.017	0.020	0.986	0.882	0.997	0.913
21:IH18	0.115	0.132	0.083	0.081	0.098	0.131	0.122	0.082	0.094	0.050	0.092	0.093	0.908	0.973	0.930	0.862
22:IR01	0.008	0.041	0.003	0.004	0.006	0.007	0.006	0.003	0.003	0.003	0.004	0.003	0.992	0.829	0.991	0.901
23:IR03	0.165	0.147	0.094	0.101	0.132	0.179	0.165	0.113	0.123	0.074	0.109	0.120	0.806	0.986	0.846	0.811
24:IR05	0.095	0.109	0.056	0.059	0.075	0.105	0.098	0.063	0.071	0.034	0.066	0.069	0.891	0.970	0.906	0.867
25:VS02	0.117	0.136	0.063	0.157	0.113	0.064	0.085	0.156	0.106	0.093	0.119	0.084	0.003	0.028	0.009	0.070
26:VS07	0.081	0.102	0.043	0.116	0.084	0.049	0.055	0.108	0.072	0.062	0.076	0.052	0.004	0.019	0.019	0.054
27:VS08	0.045	0.053	0.022	0.061	0.047	0.034	0.033	0.057	0.037	0.038	0.039	0.031	0.002	0.011	0.002	0.005
28:VS09	0.130	0.159	0.068	0.184	0.129	0.075	0.085	0.171	0.117	0.089	0.121	0.083	0.001	0.012	0.009	0.007
29:VS10	0.057	0.076	0.030	0.082	0.062	0.041	0.043	0.075	0.048	0.055	0.053	0.040	0.004	0.029	0.018	0.080
30:VS12	0.029	0.025	0.013	0.030	0.027	0.023	0.033	0.037	0.023	0.042	0.032	0.030	0.001	0.003	0.007	0.005
31:VS13	0.044	0.036	0.025	0.043	0.033	0.028	0.047	0.052	0.042	0.041	0.055	0.048	0.002	0.011	0.010	0.024
32:VS15	0.017	0.011	0.008	0.016	0.014	0.016	0.018	0.020	0.014	0.019	0.022	0.018	0.003	0.010	0.023	0.028
33:VS17	0.115	0.092	0.044	0.109	0.110	0.109	0.095	0.123	0.078	0.107	0.067	0.076	0.006	0.099	0.035	0.084
34:VS18	0.018	0.020	0.011	0.023	0.015	0.013	0.017	0.019	0.017	0.010	0.023	0.017	0.002	0.006	0.007	0.007
35:VS19	0.074	0.089	0.040	0.101	0.076	0.047	0.054	0.098	0.065	0.063	0.070	0.051	0.003	0.012	0.012	0.011
36:VS20	0.098	0.106	0.053	0.125	0.089	0.051	0.067	0.128	0.091	0.064	0.097	0.067	0.003	0.026	0.012	0.039
37:VSV02	0.035	0.029	0.011	0.032	0.030	0.040	0.030	0.035	0.022	0.033	0.019	0.024	0.007	0.031	0.012	0.037
38:VSV03	0.039	0.022	0.009	0.031	0.029	0.046	0.033	0.035	0.025	0.027	0.016	0.027	0.003	0.037	0.007	0.045
39:VSV04	0.097	0.052	0.033	0.063	0.070	0.102	0.088	0.079	0.064	0.060	0.057	0.070	0.009	0.122	0.029	0.055
40:VSV06	0.074	0.048	0.032	0.051	0.052	0.063	0.071	0.067	0.057	0.044	0.059	0.059	0.006	0.083	0.032	0.030
41:VSV07	0.097	0.057	0.033	0.066	0.075	0.104	0.085	0.078	0.059	0.071	0.057	0.066	0.017	0.123	0.044	0.065
42:VSV10	0.081	0.044	0.024	0.059	0.064	0.091	0.076	0.072	0.051	0.066	0.047	0.061	0.005	0.099	0.022	0.103
43:VSV11	0.044	0.033	0.021	0.044	0.031	0.035	0.040	0.045	0.039	0.026	0.049	0.040	0.003	0.023	0.008	0.036
44:VSV18	0.110	0.105	0.050	0.122	0.102	0.084	0.084	0.125	0.086	0.082	0.085	0.074	0.004	0.076	0.017	0.066
45:VSV19	0.057	0.035	0.022	0.044	0.042	0.052	0.054	0.053	0.041	0.042	0.046	0.047	0.004	0.066	0.014	0.077
46:VSV20	0.069	0.046	0.029	0.058	0.051	0.058	0.068	0.071	0.054	0.059	0.066	0.064	0.004	0.064	0.014	0.098
47:VB01	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
48:VB03	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001	0.001
49:VB05	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.002	0.001	0.001

Table 5-2. Pairwise relationship between *Aucklandia lappa* and related samples. Upper diagonal: Dissimilarity matrix showing the pairwise distance calculated from squared Euclidean Distance. Lower diagonal: Similarity matrix showing the pairwise similarity calculated from Cosine of vectors of variables.

(Continued)

Sample	17:IH12	18:IH13	19:IH16	20:IH17	21:IH18	22:IR01	23:IR03	24:IR05	25:VS02	26:VS07	27:VS08	28:VS09	29:VS10	30:VS12	31:VS13	32:VS15
1:AL1	3192	1961	3146	4180	2269	5035	1993	2281	1655	1737	2131	1647	1706	1714	1729	1988
2:AL11	2738	1513	2595	3649	1801	4435	1611	1809	1176	1244	1646	1159	1210	1236	1261	1514
3:AL12	3347	2106	3236	4279	2378	5089	2185	2407	1766	1825	2210	1771	1774	1765	1785	2033
4:AL15	2885	1639	2763	3811	1943	4609	1734	1947	1187	1264	1671	1163	1242	1272	1292	1547
5:AL16	3197	1959	3120	4158	2258	4989	2017	2276	1605	1674	2070	1592	1640	1658	1686	1934
6:AL17	3170	1945	3139	4171	2226	5038	1957	2254	1734	1784	2153	1729	1727	1719	1749	1987
7:AL21	3030	1804	2970	4007	2084	4861	1832	2103	1526	1596	1975	1537	1542	1523	1541	1800
8:AL22	2984	1733	2874	3920	2052	4734	1820	2055	1298	1388	1794	1288	1367	1383	1400	1661
9:AL23	3312	2055	3215	4261	2351	5090	2121	2372	1702	1782	2179	1697	1751	1754	1763	2026
10:AL25	3570	2327	3460	4500	2658	5296	2425	2662	1915	1993	2374	1933	1937	1926	1962	2215
11:AL25a	2924	1689	2815	3858	1968	4664	1768	1985	1286	1369	1763	1295	1330	1323	1331	1591
12:AL28	3263	2029	3175	4214	2310	5042	2082	2331	1691	1767	2146	1704	1715	1697	1707	1971
13:IH02	465	1431	393	129	1067	55	1572	1161	4021	4051	4416	4040	3974	3946	3981	4204
14:IH07	332	101	395	865	60	1453	30	66	1436	1478	1846	1472	1392	1390	1418	1642
15:IH08	156	939	226	20	607	119	974	711	3171	3177	3577	3185	3108	3096	3128	3316
16:IH11	354	602	442	541	442	861	592	433	2010	2065	2510	2130	1954	2039	2047	2255
17:IH12		484	171	178	230	466	420	317	2408	2439	2805	2432	2361	2341	2373	2593
18:IH13	0.913		361	988	124	1543	102	83	1207	1236	1606	1226	1160	1152	1180	1391
19:IH16	0.956	0.937		250	236	514	490	252	2249	2282	2651	2268	2204	2181	2217	2440
20:IH17	0.980	0.883	0.971		660	103	1035	736	3297	3328	3695	3316	3251	3223	3260	3482
21:IH18	0.964	0.949	0.948	0.928		1161	87	61	1535	1565	1939	1557	1486	1469	1503	1722
22:IR01	0.959	0.845	0.959	0.991	0.890		1660	1259	4061	4093	4453	4080	4016	3987	4021	4247
23:IR03	0.918	0.945	0.871	0.845	0.962	0.789		99	1337	1370	1743	1358	1289	1286	1315	1532
24:IR05	0.940	0.967	0.946	0.913	0.973	0.872	0.954		1474	1516	1892	1512	1430	1428	1459	1682
25:VS02	0.010	0.029	0.010	0.004	0.016	0.003	0.029	0.030		123	318	143	243	285	294	297
26:VS07	0.009	0.029	0.008	0.004	0.016	0.003	0.026	0.020	0.858		217	272	118	327	286	138
27:VS08	0.006	0.017	0.004	0.001	0.006	0.003	0.013	0.008	0.781	0.864		605	305	576	491	298
28:VS09	0.005	0.025	0.007	0.002	0.011	0.002	0.023	0.012	0.831	0.689	0.537		405	299	462	512
29:VS10	0.011	0.032	0.010	0.004	0.019	0.003	0.032	0.030	0.692	0.859	0.798	0.494		330	255	263
30:VS12	0.003	0.009	0.003	0.001	0.006	0.001	0.006	0.004	0.623	0.587	0.545	0.614	0.532		294	399
31:VS13	0.006	0.017	0.004	0.002	0.009	0.002	0.014	0.010	0.628	0.653	0.628	0.426	0.656	0.585		353
32:VS15	0.008	0.025	0.005	0.003	0.012	0.003	0.018	0.011	0.720	0.877	0.797	0.510	0.750	0.597	0.656	
33:VS17	0.049	0.097	0.031	0.022	0.071	0.006	0.124	0.081	0.660	0.584	0.396	0.799	0.448	0.632	0.377	0.510
34:VS18	0.005	0.011	0.003	0.001	0.006	0.002	0.009	0.005	0.612	0.619	0.639	0.462	0.598	0.727	0.601	0.661
35:VS19	0.011	0.021	0.005	0.003	0.012	0.004	0.019	0.009	0.833	0.833	0.762	0.715	0.684	0.753	0.578	0.785
36:VS20	0.015	0.030	0.009	0.006	0.019	0.003	0.034	0.024	0.320	0.271	0.147	0.275	0.258	0.496	0.240	0.202
37:VSV02	0.023	0.063	0.011	0.005	0.016	0.012	0.047	0.028	0.539	0.694	0.760	0.286	0.770	0.461	0.623	0.689
38:VSV03	0.013	0.059	0.014	0.006	0.017	0.006	0.048	0.034	0.580	0.698	0.843	0.267	0.714	0.367	0.603	0.682
39:VSV04	0.070	0.127	0.037	0.027	0.084	0.012	0.153	0.094	0.231	0.216	0.230	0.247	0.370	0.320	0.329	0.185
40:VSV06	0.049	0.081	0.027	0.020	0.066	0.005	0.104	0.063	0.228	0.271	0.142	0.231	0.349	0.286	0.421	0.277
41:VSV07	0.091	0.152	0.036	0.028	0.089	0.024	0.167	0.096	0.321	0.364	0.206	0.304	0.440	0.381	0.395	0.353
42:VSV10	0.044	0.099	0.032	0.020	0.063	0.007	0.118	0.085	0.499	0.369	0.303	0.501	0.308	0.509	0.297	0.309
43:VSV11	0.012	0.037	0.008	0.004	0.013	0.005	0.030	0.021	0.589	0.522	0.516	0.436	0.431	0.641	0.436	0.521
44:VSV18	0.037	0.066	0.022	0.016	0.050	0.005	0.089	0.062	0.679	0.585	0.533	0.694	0.429	0.717	0.434	0.480
45:VSV19	0.033	0.057	0.020	0.014	0.043	0.005	0.074	0.058	0.455	0.494	0.556	0.274	0.627	0.460	0.640	0.499
46:VSV20	0.026	0.056	0.020	0.012	0.040	0.004	0.069	0.058	0.546	0.518	0.466	0.393	0.650	0.520	0.741	0.483
47:VB01	0.001	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.002
48:VB03	0.001	0.002	0.000	0.000	0.001	0.000	0.002	0.001	0.006	0.013	0.004	0.010	0.010	0.013	0.008	0.015
49:VB05	0.001	0.002	0.000	0.000	0.001	0.000	0.002	0.001	0.002	0.002	0.001	0.002	0.002	0.002	0.002	0.002

Table 5-2. Pairwise relationship between *Aucklandia lappa* and related samples. Upper diagonal: Dissimilarity matrix showing the pairwise distance calculated from squared Euclidean Distance. Lower diagonal: Similarity matrix showing the pairwise similarity calculated from Cosine of vectors of variables.

(Continued)

Sample	33:VS17	34:VS18	35:VS19	36:VS20	37:VSV02	38:VSV03	39:VSV04	40:VSV06	41:VSV07	42:VSV10	43:VSV11	44:VSV18	45:VSV19	46:VSV20	47:VB01	48:VB03	49:VB05
1:AL1	1629	1904	1729	2232	1929	2137	1724	1766	1649	1657	1877	1642	1850	1637	7837	5129	5878
2:AL11	1199	1417	1243	1774	1461	1692	1324	1335	1234	1232	1418	1190	1409	1190	7347	4639	5390
3:AL12	1762	1945	1810	2371	2002	2229	1857	1864	1771	1767	1947	1761	1939	1718	7866	5159	5910
4:AL15	1218	1454	1266	1770	1497	1716	1349	1370	1263	1255	1442	1208	1437	1217	7388	4681	5431
5:AL16	1581	1850	1669	2199	1879	2099	1710	1744	1624	1623	1842	1598	1817	1602	7777	5069	5819
6:AL17	1636	1909	1768	2346	1917	2118	1714	1781	1637	1641	1890	1679	1856	1649	7834	5124	5873
7:AL21	1481	1719	1577	2131	1755	1966	1560	1591	1487	1483	1701	1502	1672	1456	7650	4942	5692
8:AL22	1314	1579	1384	1872	1611	1827	1443	1465	1365	1357	1559	1317	1541	1320	7511	4804	5554
9:AL23	1715	1936	1774	2280	1984	2199	1809	1825	1736	1731	1917	1709	1908	1689	7870	5162	5913
10:AL25	1863	2150	1973	2538	2164	2391	2010	2043	1914	1905	2140	1909	2104	1879	8071	5364	6115
11:AL25a	1321	1507	1358	1878	1571	1796	1408	1411	1326	1321	1487	1305	1486	1261	7444	4736	5486
12:AL28	1671	1889	1750	2292	1935	2145	1752	1775	1679	1669	1868	1680	1850	1628	7822	5115	5865
13:IH02	3978	4123	4030	4655	4166	4404	4052	4065	3944	3954	4138	3990	4131	3914	10032	7325	8077
14:IH07	1312	1564	1467	2056	1583	1791	1349	1408	1275	1290	1558	1348	1488	1295	7477	4769	5515
15:IH08	3081	3273	3173	3785	3319	3555	3172	3169	3057	3083	3291	3127	3270	3057	9193	6480	7236
16:IH11	1957	2215	2118	2660	2213	2406	2077	2126	1981	1905	2178	1991	2095	1880	8131	5421	6174
17:IH12	2304	2515	2416	3022	2535	2777	2339	2384	2224	2290	2521	2332	2473	2278	8427	5719	6469
18:IH13	1096	1326	1226	1823	1313	1531	1131	1192	1028	1071	1311	1138	1280	1079	7242	4533	5285
19:IH16	2178	2361	2268	2881	2402	2618	2246	2269	2162	2153	2369	2200	2342	2131	8271	5564	6314
20:IH17	3223	3402	3309	3924	3452	3673	3290	3310	3203	3202	3416	3244	3385	3177	9309	6603	7352
21:IH18	1428	1648	1550	2154	1689	1910	1482	1512	1397	1415	1657	1462	1605	1404	7561	4852	5602
22:IR01	4020	4163	4070	4694	4193	4434	4084	4109	3968	3991	4173	4029	4169	3954	10071	7365	8117
23:IR03	1187	1460	1359	1942	1462	1673	1216	1286	1130	1173	1450	1236	1382	1192	7375	4666	5415
24:IR05	1374	1606	1511	2100	1628	1836	1427	1476	1347	1346	1600	1404	1540	1340	7518	4810	5558
25:VS02	270	364	141	1045	462	547	675	682	534	386	395	258	517	333	6833	4114	4876
26:VS07	345	367	146	1127	318	412	712	666	521	505	471	347	495	370	6861	4127	4905
27:VS08	749	499	340	1589	347	253	989	1101	953	838	665	599	609	651	7227	4509	5271
28:VS09	163	510	244	1112	720	917	671	689	558	392	546	251	697	452	6847	4120	4890
29:VS10	413	362	252	1101	234	393	526	547	414	499	521	431	344	239	6786	4060	4829
30:VS12	264	246	191	799	504	755	545	576	437	337	327	205	477	309	6751	4019	4795
31:VS13	468	361	338	1125	370	514	561	489	450	510	518	430	333	178	6790	4068	4834
32:VS15	492	380	232	1332	365	455	867	773	638	668	544	524	567	493	7014	4268	5057
33:VS17		562	316	1089	664	884	542	572	391	279	542	269	646	426	6795	4055	4837
34:VS18	0.375		144	629	472	615	768	796	612	473	220	319	581	387	6932	4216	4975
35:VS19	0.608	0.850		714	348	519	625	651	459	353	225	185	503	382	6840	4111	4880
36:VS20	0.271	0.637	0.571		1156	1528	1087	1124	941	841	490	715	1178	982	7469	4746	5513
37:VSV02	0.309	0.567	0.659	0.303		166	429	540	375	549	500	523	265	382	6986	4265	5031
38:VSV03	0.268	0.545	0.605	0.177	0.891		736	881	741	753	630	668	489	580	7219	4507	5262
39:VSV04	0.357	0.214	0.295	0.307	0.589	0.432		158	171	265	508	580	400	396	6877	4161	4920
40:VSV06	0.325	0.190	0.270	0.282	0.484	0.315	0.830		210	377	527	658	494	437	6883	4153	4925
41:VSV07	0.478	0.312	0.424	0.386	0.617	0.395	0.799	0.755		265	463	489	475	367	6778	4046	4823
42:VSV10	0.619	0.462	0.548	0.463	0.419	0.376	0.681	0.545	0.634		283	311	551	383	6771	4058	4813
43:VSV11	0.412	0.790	0.770	0.732	0.549	0.538	0.492	0.475	0.497	0.696		307	606	478	6950	4233	4994
44:VSV18	0.649	0.652	0.773	0.568	0.464	0.466	0.318	0.230	0.354	0.579	0.675		551	352	6804	4074	4848
45:VSV19	0.292	0.443	0.475	0.270	0.760	0.646	0.598	0.505	0.480	0.382	0.430	0.403		223	6947	4235	4992
46:VSV20	0.383	0.548	0.485	0.333	0.594	0.532	0.496	0.447	0.462	0.422	0.450	0.497	0.761		6728	4017	4771
47:VB01	0.001	0.001	0.001	0.001	0.002	0.000	0.001	0.001	0.004	0.000	0.001	0.001	0.000	0.000		389	195
48:VB03	0.016	0.005	0.011	0.005	0.008	0.002	0.006	0.011	0.017	0.004	0.006	0.011	0.003	0.003	0.998		44
49:VB05	0.002	0.001	0.003	0.001	0.002	0.001	0.002	0.002	0.005	0.002	0.002	0.001	0.001	0.001	0.998	0.999	

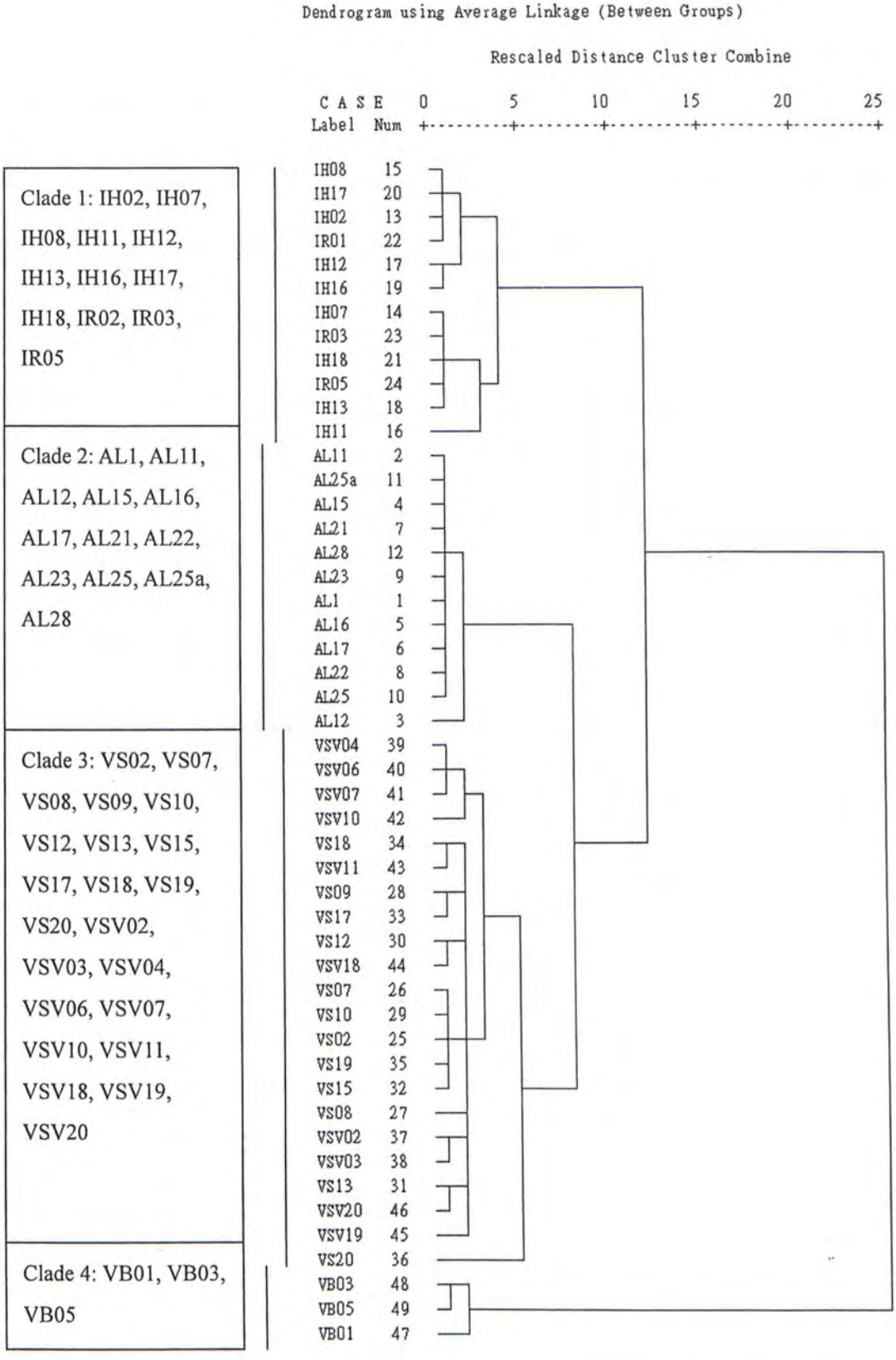

Figure 5-2. Dendrogram calculated by square Euclidean distance method with between-groups linkage. Sample codes are listed on the left, AL: *Aucklandia lappa*, IH: *Inula helenium*, IR: *Inula racemosa*, VB: *Vladimiria berardioides*, VS: *Vladimiria soliei*, VSV: *V. soliei* var. *cinerea*.

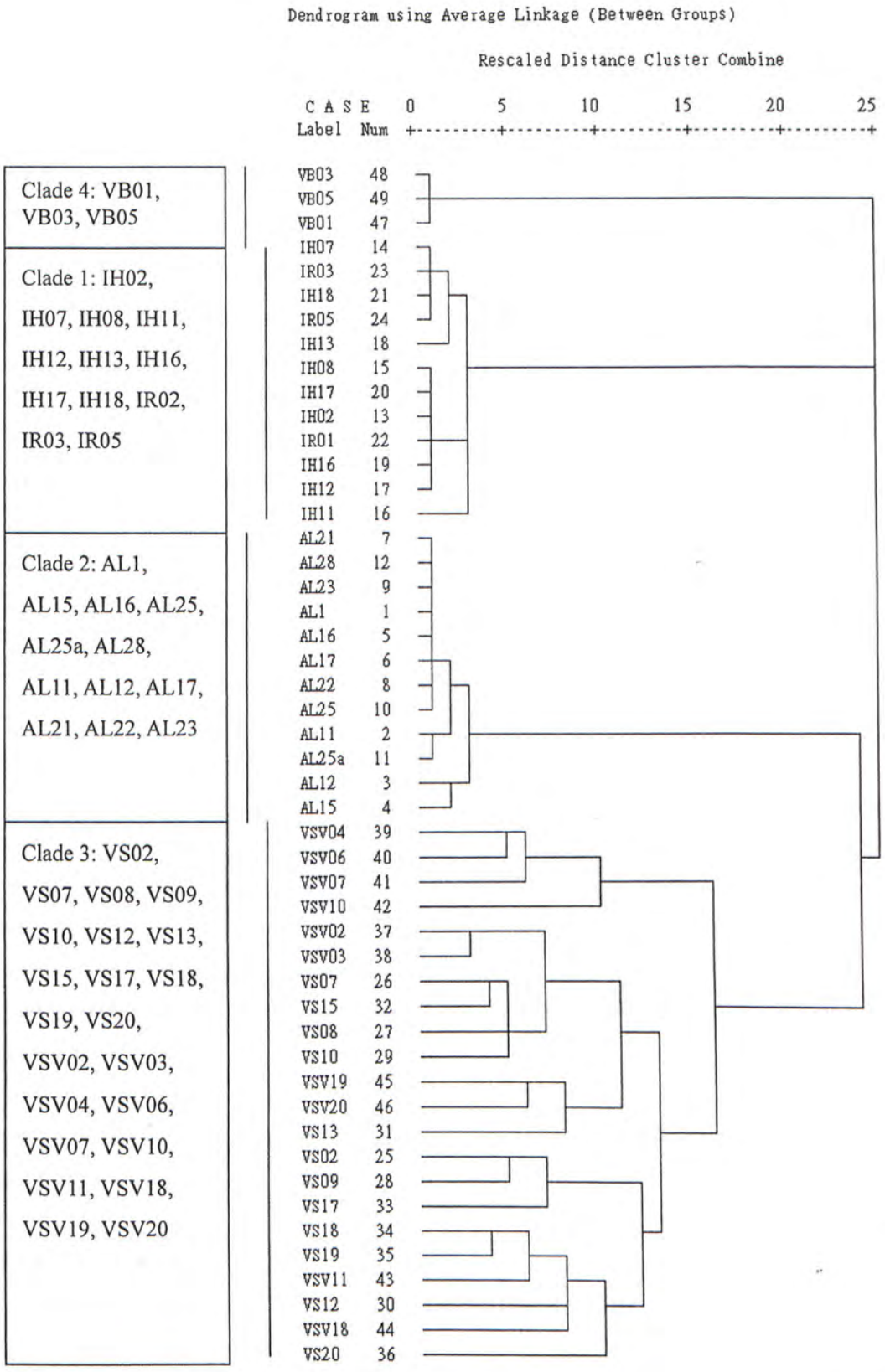

Figure 5-3. Dendrogram calculated by cosine of vector with between-groups linkage. Sample codes are listed on the left, AL: *Aucklandia lappa*, IH: *Inula helenium*, IR: *Inula racemosa*, VB: *Vladimiria berardioides*, VS: *Vladimiria soliei*, VSV: *V. soliei* var. *cinerea*.

Table 5-3. The chemicals detected in GC-MS analysis of *Aucklandia lappa*.

R.T.	m/z	Identity	Undetectable in other species
12.18	154	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	Yes
18.50	150	Thymol	
22.79	194	3-Buten-2-ol, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-	
23.22	204	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, (1 ¢ ,2 £ ,4 £)-	
23.70	204	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	
24.44	204	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-	Yes
25.08	204	Unknown	
25.15	194	2-Butanone, 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-	
25.28	204	Caryophyllene	
25.73	192	Unknown	
26.07	204	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	Yes
27.11	204	4,7,10-Cycloundecatriene, 1,1,4,8-tetramethyl-, cis, cis, cis-	
27.12	194	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	
27.87	204	Spiro[4.5]dec-7-ene, 1,8-dimethyl-4-(1-methylethenyl)-,[1S-(1 ¢ ,4 £ ,5 ¢)]-	Yes
27.92	204	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1 ¢ ,4a ¢ ,8a ¢)-	Yes
28.30	204	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	
28.73	204	1H-Benzocycloheptene, 2,4a,5,6,7,8-hexahydro-3,5,5,9-tetramethyl-, (R)-	
28.98	202	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	Yes
29.04	192	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-, (E)-	
29.52	210	1-Pentadecene	
29.71	204	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-, [2R-(2 ¢ ,4a ¢ ,8a £)]-	
29.92	204	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a-octahydronaphthalene	Yes
30.34	208	4-(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-butan-2-one	
30.70	204	Unknown	Yes
31.14	220	Unknown	Yes
31.65	220	Unknown	Yes
31.83	204	Unknown	
32.24	204	Unknown	
32.45	204	Cyclohexanemethanol, 4-ethenyl- ¢ , ¢ ,4-trimethyl-3-(1-methylethenyl)-, [1R-(1 ¢ ,3 ¢ ,4 £)]-	
33.20	208	Unknown	
33.54	222	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-, (E)-	

Table 5-3. The chemicals detected in GC-MS analysis of *Aucklandia lappa*.

R.T.	m/z	Identity	Undetectable in other species
(Continued)			
33.87	264	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	
34.09	278	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalen-2-yl ester	
34.19	220	Caryophyllene oxide	
34.62	220	Diepicedrene-1-oxide	
34.99	220	Longifolenaldehyde	
35.27	220	Isoaromadendrene epoxide	
35.72	220	12-Oxabicyclo[9.1.0]dodeca-3,7-diene, 1,5,5,8-tetramethyl-, [1R-(1R*,3E,7E,11R*)]-	
36.18	222	Unknown	
37.07	204	1H-Cyclopropa[a]naphthalene, 1a,2,3,3a,4,5,6,7b-octahydro-1,1,3a,7-tetramethyl-, [1aR-(1a ¢ ,3a ¢ ,7b ¢)]-	Yes
37.63	222	2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octahydro- ¢ , ¢ ,4a,8-tetramethyl-, [2R-(2 ¢ ,4a £ ,8 £)]-	Yes
37.91	222	1-Naphthalenol, decahydro-1,4a-dimethyl-7-(1-methylethylidene)-, [1R-(1 ¢ ,4a £ ,8a ¢)]-	
38.20	222	Unknown	Yes
39.17	264	Unknown	
40.05	218	Unknown	
40.19	218	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4,4a-dimethyl-6-(1-methylethenyl)-, [4R-(4 ¢ ,4a ¢ ,6 £)]-	
40.37	236	Bicyclo[4.4.0]dec-5-ene, 1,5-dimethyl-3-hydroxy-8-(1-methylene-2-hydroxyethyl-1)-	Yes
40.77	220	Bergamotol, Z- ¢ -trans-	Yes
40.89	222	Cyclohexane, 1-(cyclohexylmethyl)-4-(1-methylethyl)-	
41.04	220	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	
41.43	220	Unknown	
41.52	220	Unknown	Yes
41.67	220	Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl-	
41.70	220	Longipinocarveol, trans-	
42.84	220	Unknown	
43.05	220	Cedren-13-ol, 8-	
43.16	220	2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-naphthalen-2-yl)-prop-2-en-1-ol	Yes
43.81	220	Cedren-13-ol, 8-	
44.33	188	Bicylo[4.1.0]heptane, 7-bicyclo[4.1.0]hept-7-ylidene-	
44.56	220	Unknown	Yes

Table 5-3. The chemicals detected in GC-MS analysis of *Aucklandia lappa*.

R.T.	m/z	Identity	Undetectable in other species
(Continued)			
45.89	232	Cyclodeca[b]furan-2(3H)-one, 3a,4,5,8,9,11a-hexahydro-6,10-dimethyl-3-methylene-, [3aS-(3aR*,6E,10E,11aS*)]-(Costunolide)	Yes
46.03	248	Methyl aciphyllate	
46.72	176	11-Methylene-tricyclo[5.3.1.1(2,6)]dodecane	Yes
46.98	162	1,4,8-Dodecatriene, (E,E,E)-	
47.43	232	2(3H)-Benzofuranone, 6-ethenylhexahydro-6-methyl-3-methylene-7-(1-methylethenyl)-, [3aS-(3a ¢ ,6 ¢ ,7 £ ,7a £)]-	
47.89	232	Unknown	
48.53	262	9 £ -Acetoxy-3,5,8-trimethyltricyclo[6.3.1.0(1,5)]dodec-2-ene	Yes
49.06	216	Musizin	Yes
49.45	262	Unknown	
49.68	262	9 £ -Acetoxy-3,4,8-trimethyltricyclo[6.3.1.0(1,5)]dodec-3-ene	Yes
50.28	230	Unknown	Yes
50.83	244	Unknown	Yes
50.88	232	Unknown	Yes
51.01	268	Unknown	
51.55	232	Eudesma-5,11(13)-dien-8,12-olide	Yes
52.02	234	1,2-Longidione	
52.78	232	Unknown	Yes
52.83	232	Unknown	
53.61	232	Cyclodeca[b]furan-2(3H)-one, 3a,4,5,8,9,11a-hexahydro-3,6,10-trimethyl-, [3S-(3R*,3aR*,6E,10E,11aR*)]- (Dihydrocostuslactone)	
53.87	256	n-Hexadecanoic acid	
54.92	346	Unknown	
55.60	230	Azuleno[4,5-b]furan-2(3H)-one, decahydro-3,6,9-tris(methylene)-, [3aS-(3a ¢ ,6a ¢ ,9a ¢ ,9b £)]- (Dehydrocostuslactone)	
55.75	284	Hexadecanoic acid, ethyl ester	
56.87	250	16-Methyloxacyclohexadeca-3,5-dien-2-one	
60.34	296	Unknown	

5.3.4 Purification of chemical markers from *Aucklandia lappa*

Two chemicals, dehydrocostuslactone (R.T. = 55.60) and costunolide (R.T. = 45.89) were purified from *Aucklandia lappa* with amounts of 1.03g and 0.39g respectively. Their purity and identities were confirmed by GC-MS analysis. The gas chromatogram and mass spectrum scan of dehydrocostuslactone are shown in Figure 5-4 and Figure 5-5, and those of costunolide are shown in Figure 5-6 and Figure 5-7.

5.3.5 Standardization of the purified chemical markers

Serial dilutions of the purified chemical markers were analyzed by GC, and standardization curves were plotted by mass of chemical injected against peak abundance as shown in Figure 5-8 and Figure 5-9. The response factor (Rf) of dehydrocostuslactone and costunolide were calculated from the graphs and were 4.39×10^{-9} and 5.51×10^{-9} respectively where

Mass of chemical (ng) injected into GC = Rf × Peak abundance

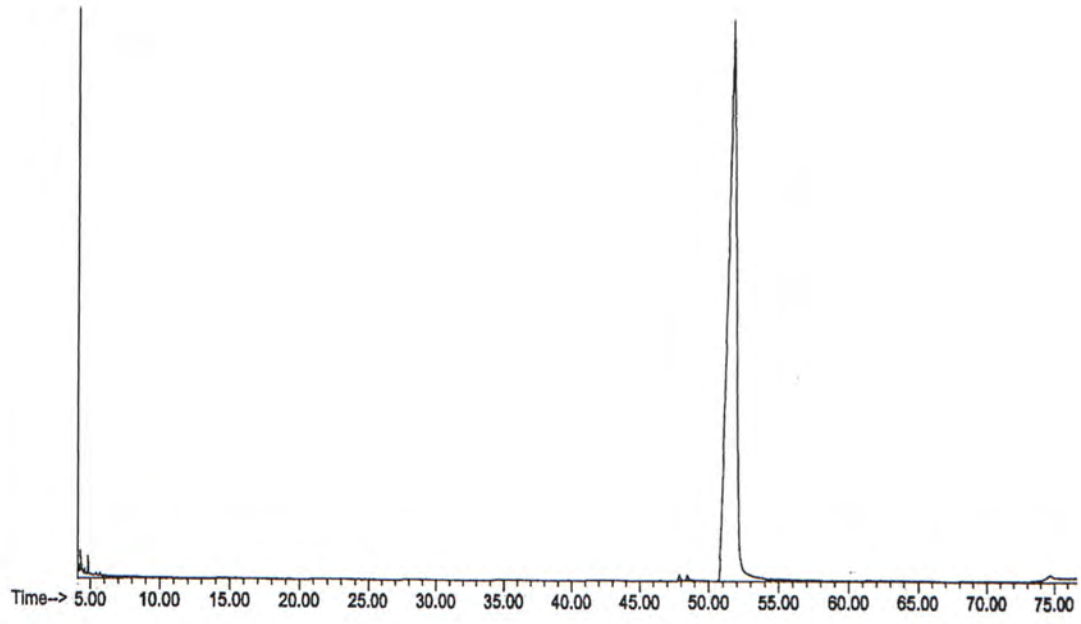

Figure 5-4. Gas chromatographic analysis of purified dehydrocostuslactone

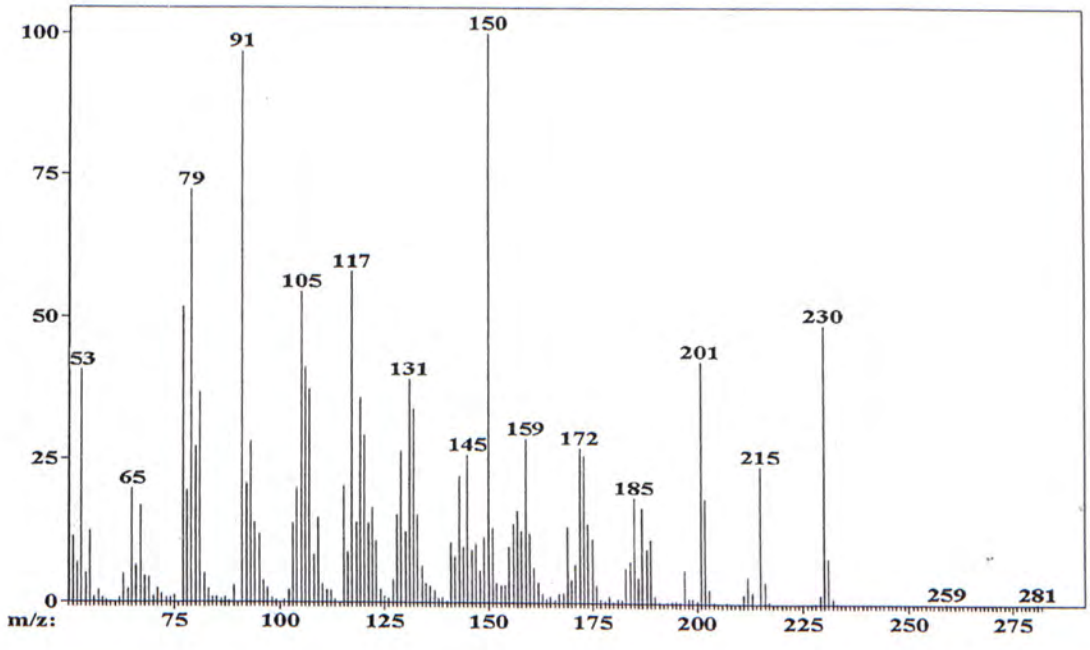

Figure 5-5. Mass spectrum scan of purified dehydrocostuslactone

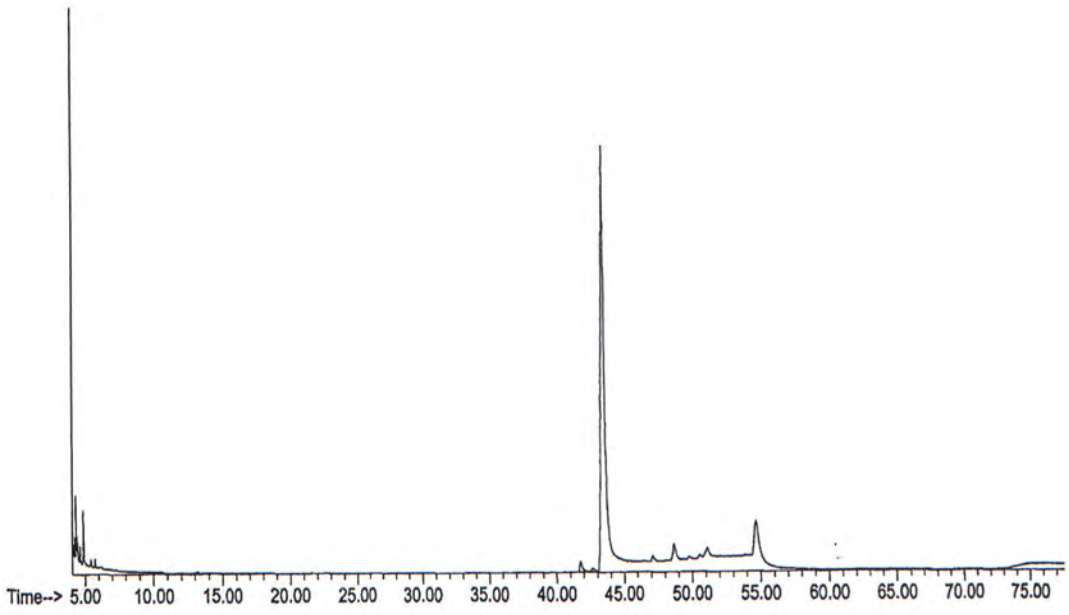

Figure 5-6. Gas chromatographic analysis of purified costunolide

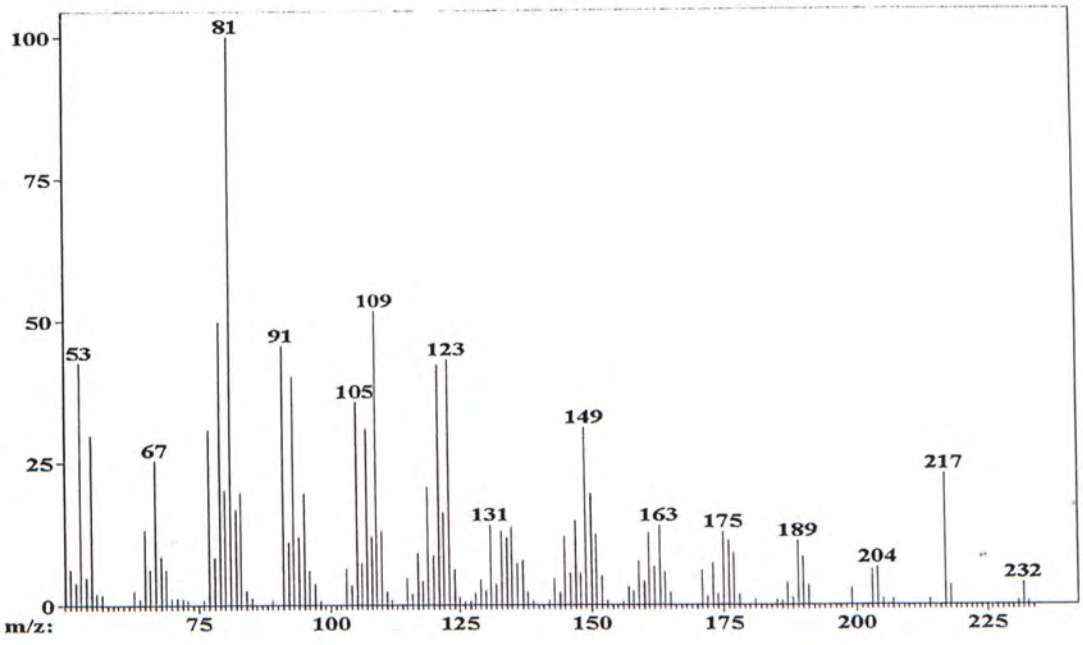

Figure 5-7. Mass spectrum scan of purified costunolide

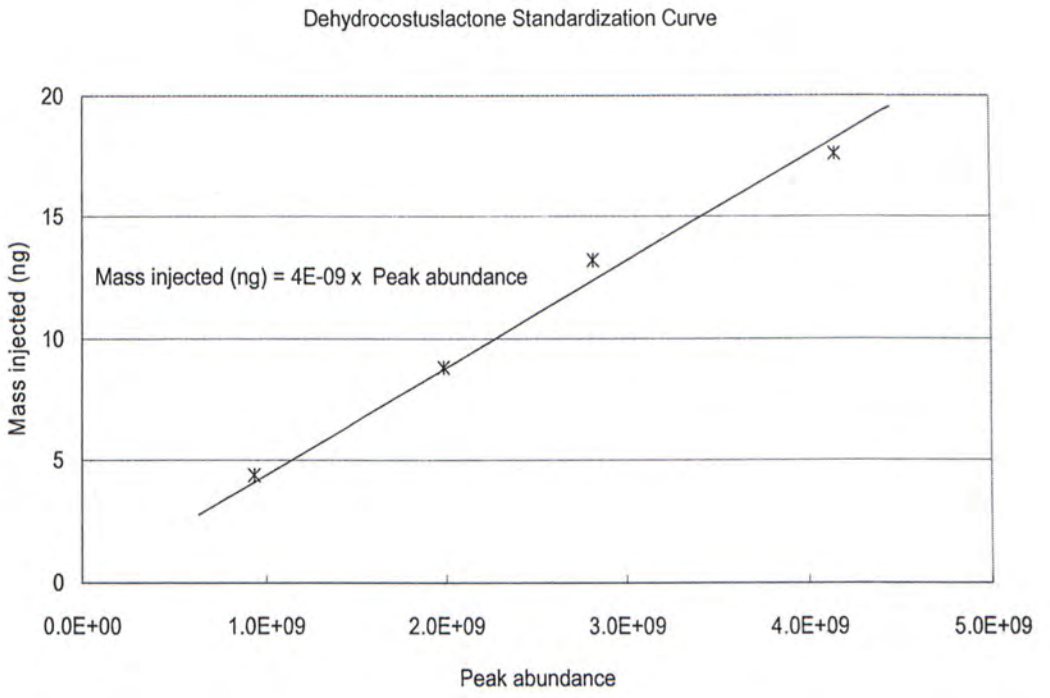

Figure 5-8. Standardization curve of dehydrocostuslactone

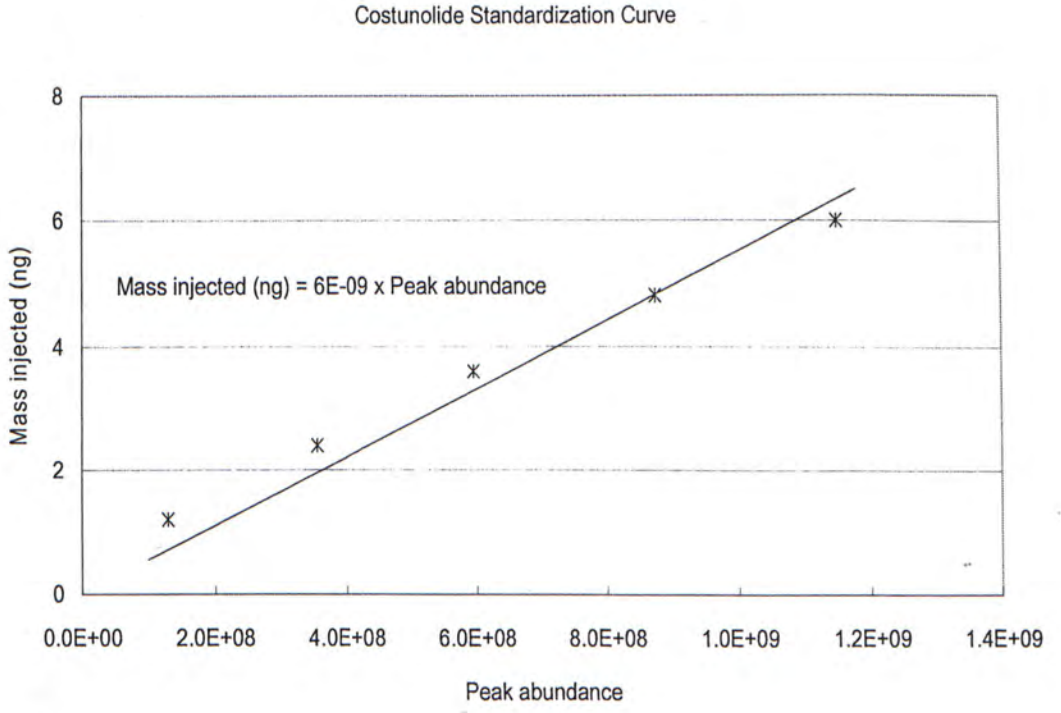

Figure 5-9. Standardization curve of costunolide

5.3.6 Quantitative analysis of chemical markers

27 samples of *Aucklandia lappa* were analyzed by GC and the peak abundances of the markers were recorded. The peak abundances were used to calculate the content of the corresponding chemicals. The percentage of dehydrocostuslactone in total essential oil ranged from 9.7% to 87.3% with an average of 45.6% and that of costunolide ranged from 3.1% to 24.8% with an average of 9.1%. The percentage of dehydrocostuslactone and costunolide in total essential oil ranged from 23.9% to 97% with an average of 54.6%. Details of individual samples are listed in Table 5-4.

Table 5-4. The content of essential oil and marker chemicals in *Aucklandia lappa* samples

Sample	Amount of extracted essential oil (g)	Amount of dehydro-costuslactone extracted /mg (Percentage in total essential oil)	Amount of costunolide extracted /mg (Percentage in total essential oil)	Percentage of dehydrocostus-lactone and costunolide in total essential oil
AL-1	0.415	362 (87.3)	40 (9.7)	97.0
AL-2	0.226	112 (49.5)	38 (16.9)	66.4
AL-3	0.310	153 (49.3)	17 (5.6)	55.0
AL-4	0.200	92 (46.0)	14 (6.9)	52.9
AL-5	0.200	102 (51.1)	19 (9.6)	60.7
AL-6	0.208	63 (30.2)	13 (6.2)	36.4
AL-7	0.090	9 (9.7)	13 (14.2)	23.9
AL-8	0.288	65 (22.6)	10 (3.5)	26.2
AL-9	0.270	104 (38.5)	11 (3.9)	42.4
AL-10	0.170	61 (35.8)	42 (24.8)	60.6
AL-11	0.232	165 (71.2)	24 (10.3)	81.5
AL-12	0.180	42 (23.1)	31 (17.5)	40.6
AL-13	0.190	87 (46.0)	12 (6.1)	52.1
AL-14	0.340	127 (37.3)	13 (3.7)	41.0
AL-15	0.178	129 (72.6)	21 (12.1)	84.6
AL-16	0.170	114 (67.2)	15 (9.0)	76.3
AL-18	0.508	183 (36.0)	29 (5.7)	41.7
AL-19	0.266	106 (39.7)	14 (5.2)	44.9
AL-20	0.251	179 (71.2)	38 (15.1)	86.3
AL-21	0.320	73 (22.8)	10 (3.1)	26.0
AL-22	0.260	75 (28.8)	19 (7.5)	36.3
AL-23	0.202	137 (67.6)	15 (7.4)	75.0
AL-25	0.382	156 (40.9)	17 (4.6)	45.5
AL-25a	0.320	156 (48.7)	61 (19.2)	67.9
AL-26	0.160	59 (36.9)	7 (4.3)	41.1
AL-27	0.360	259 (71.9)	30 (8.4)	80.3
AL-28	0.191	55 (28.6)	7 (3.9)	32.5

5.4 Discussion

5.4.1 Analysis of chemical composition

GC-MS gave a very high resolution of the complex chemical composition of *A. lappa* and related species. Major peaks were eluted from retention time 20min to 60min. 90 chemicals were detected in the essential oil extract of *A. lappa*, 28 of which were found to be unique to *A. lappa*. 91 essential oil components were identified from *Inula* (including *I. helenium* and *I. racemosa*) and 41 were unique. 132 chemicals were identified from *Vladimiria soliei* (including *V. soliei* var. *cinerea*) and 82 were unique. 57 chemicals were detected in *V. berardioidea*, 21 of which were unique.

Benefited from the mass spectrometry, chemicals with very near retention times could still be distinguished and that eliminated the possibility that different chemicals were mistakenly aligned as one chemical.

5.4.2 A comparison on chemometric methods

Two methods of calculation were used to generate the pairwise relationships between the samples. Cosine of vectors of variables was used to calculate similarity values

which range from 0 to 1, and the higher the value, the higher the similarity it has between the two samples. This method emphasizes on the clustering of closely-related samples. Squared Euclidean distance was used to calculate the dissimilarity values in which larger values means larger distances between samples. In contrast, it focuses on the clustering of less-related samples.

In the analysis of GC data in this study, every peak was normalized within the sample, and the two calculations gave results which are essentially representing the same thing – except that HCA with square Euclidean distance is better in dealing with samples with larger variations and that with cosine of vectors is better in dealing with samples with smaller variations.

Using cosine of vectors in HCA has the advantage of using raw GC data as the concentration difference (represented by the vector length) is not taken into account for the calculation of the similarities, and it is convenient to apply of GC-MS for authentication. In contrast, HCA with square Euclidean distance requires normalized data before analysis.

5.4.3 Similarity of chemical profiles

Table 5-5. A summary of ranges of similarity and dissimilarity value between *A. lappa* samples and related species. Upper: similarity values. The possible value ranges from 0 to 1. The larger the value, the more similar it is between two samples. Lower. Dissimilarity values. The smallest possible value is 0. The smaller the value, the more similar it is between two samples.

Sample	*A. lappa*	*I. helenium*	*I. racemosa*	*V. souliei*	*V. souliei* var. *cinerea*	*V. berardioidea*
A. lappa	0.853-0.993; 23-462					
I. helenium	0.003-0.147; 1513-5253	0.814-0.997; 20-1431				
I. racemosa	0.003-0.179; 1611-5296	0.806-0.992; 30-1572	0.789-0.954; 99-1660			
V. souliei	0.008-0.184; 1159-2538	0.001-0.099; 1096-4655	0.001-0.124; 1187-4694	0.147-0.877; 118-1589		
V. souliei var. *cinerea*	0.009-0.135; 1190-2391	0.003-0.152; 1208-4404	0.004-0.167; 1130-4434	0.142-0.843; 178-1528	0.230-0.891; 158-881	
V. berardioidea	0.000-0.002; 4638-8071	0.000-0.002; 4533-10032	0.000-0.002; 4666-10071	0.000-0.016; 4019-7469	0.000-0.017; 4017-7219	0.998-0.999; 44-389

In the analysis of essential oil content, both calculations showed consistent results (Table 5-5) in which higher similarity values from cosine calculation always came with low dissimilarity values from squared Euclidean distance. Samples of *A. lappa* showed a much higher intra-specific similarity than inter-specific one. Samples from *I. helenium* and *I. racemosa* were not distinguishable, but they still had a lower similarity towards other species. *V. soliei* and its variety *V. souliei* var. *cinerea* could not be discriminated neither. However, *V. berardioides* were distant

from other samples despite being the same genus of *Vladimiria*.

5.4.4 Dendrogram analysis

Hierarchical cluster analysis can give a graphic presentation called dendrogram which shows the hierarchical relationship among samples. The dendrogram from GC-MS can also be used to infer phylogenetic relationships among species like the phylograms shown in the previous chapters.

The dendrograms were nicely divided into clades. Square Euclidean distance method (Figure 5-2) which focuses on the less-related samples can be used to reveal relationships in higher taxonomic levels. It showed that *A. lappa* was more closely related to *V. soliei* (with *V. souliei* var. *cinerea)* than to *Inula*. That is consistent with the modern classification in Asteraceae (Section 1.13). However, clade of *V. berardioides* was not clustered with *Vladimiria*. This should be considered in the review of classification in *Vladimiria*. Neither *I. helenium* and *I. racemosa* nor *V. soliei* and *V. souliei* var. *cinerea* were clustered in separate clades. These showed a close chemical profiles for the two pairs of species.

In cosine of vectors method (Figure 5-3), relationship of samples of higher similarity

was revealed. Samples of *A. lappa* showed a short distance in the cluster, suggesting that chemical profiles were similar among *A. lappa*. Samples with less homogenous chemical profiles were also shown in the clade and the samples included AL11, AL12, AL15 and AL25a. However, *V. soliei* and *V. souliei* var. *cinerea* showed greater deviation in chemical profiles and they did not group into separate clades, and this was also shown in their distance value in Table 5-2. It revealed a high variation in chemical contents within the two species. So far, environmental factors and post-harvest processing are unlikely related to this variation. This high variation in the essential oil centents of *V. soliei* (including *V. souliei* var. *cinerea*) might be due to individual variations. Although GC-MS could not be used to distinguish the two groups, it still supported the grouping in the *Pharmacopoeia of People's Republic of China Volume I* that *V. soliei* and *V. souliei* var. *cinerea* are grouped to the same medicinal material Chuanmuxiang. Similar to the results from square Euclidean distance method, clade of *V. berardioides* in cosine of vectors method was not clustered with *Vladimiria* but far away from other samples.

5.4.5 Utility of GC-MS in authentication of *A. lappa* and related species

GC-MS analysis gives highly consistent and repeatable results, making it an excellent technique in authentication of TCM, especially for those which are rich in essential oils. *A. lappa* was well separated from other species in the dendrograms and *V. soliei* including its variety form clade on its own, which means that the two species can be authenticated by GC-MS. *Inula helenium*, however, formed a cluster with *I. racemosa*, which indicates the two species are very similar in terms of volatile chemicals.

GC-MS revealed the difference of the three medicinal species and therefore, their use should not be inter-changed. GC-MS can assess *A. lappa* and related species efficiently and effectively in terms of separation of their volatile components which are highly correlated to their pharmacological activities, and this technique should be routinely carried out to monitor the quality of medicinal materials on the market.

5.4.6 Limitations

Although GC-MS gave peaks which were unique to samples of the species, it is possible that some of the chemicals detected by GC were due to bacterial or fungal contamination. The root parts of the medicinal materials which have close contact

with soil are expected to have different species of bacteria or fungi. Lambais *et al.* (2006) showed that bacterial communities from the same plant species varied. The difference in chemical composition, especially components in minute amount within a species as shown in the chromatographs, can be caused by bacteria or fungi. This limitation can affect the results of GC-MS used for phylogenetic purpose. In this study, the effect of bacterial or fungal contamination appeared to be not serious because HCA can group species into their respective clades.

The chemical identification (Table 5-3) was achieved by NIST98 mass spectral database, but it is only a preliminary identification. For more accurate chemical identities, further confirmation such as by Nuclear Magnetic Resonance is required. Moreover, some chemicals could not be identified by matching to NIST98 database. Newer version of database may be obtained for a more comprehensive analysis of the chemical compounds. However, this would not affect peak alignment which was accomplished by direct comparison of mass spectra. Therefore, the accuracy of HCA is not affected.

5.4.7 Comparison with molecular data

Previously, the molecular authentication of *Aucklandia lappa* and related species were also applied (Chen, 2004) and a comparison is made here. In the UPGMA phylogram from nrDNA 5S spacer (Figure 5-10 upper) and ITS2 (Figure 5-10 lower), three groups were established: *Aucklandia lappa*, *Vladimira* and *Inula*. This supports the grouping in the two dendrograms of the three species (Figure 5-2 and Figure 5-3). This shows that both chemical and molecular methods can be used in authentication of the species.

Also, *Vladimira souliei* were clustered with its variety *V. souliei* var *cinerea*, so were *Inula helenium* and *I. racemosa*, as shown in both molecular and chemical data. However, the clustering of *V. berardioides* as shown in molecular and chemical data is different: *V. berardioides* was clustered with other *Vladimira* in 5S spacer phylogram, but it was not clustered with any group in both square Euclidean distance method (Figure 5-2) and cosine of vectors method (Figure 5-3). To resolve this discrepancy, studies such as phylogenetic studies of other sequence markers or mophorlogical analysis are required.

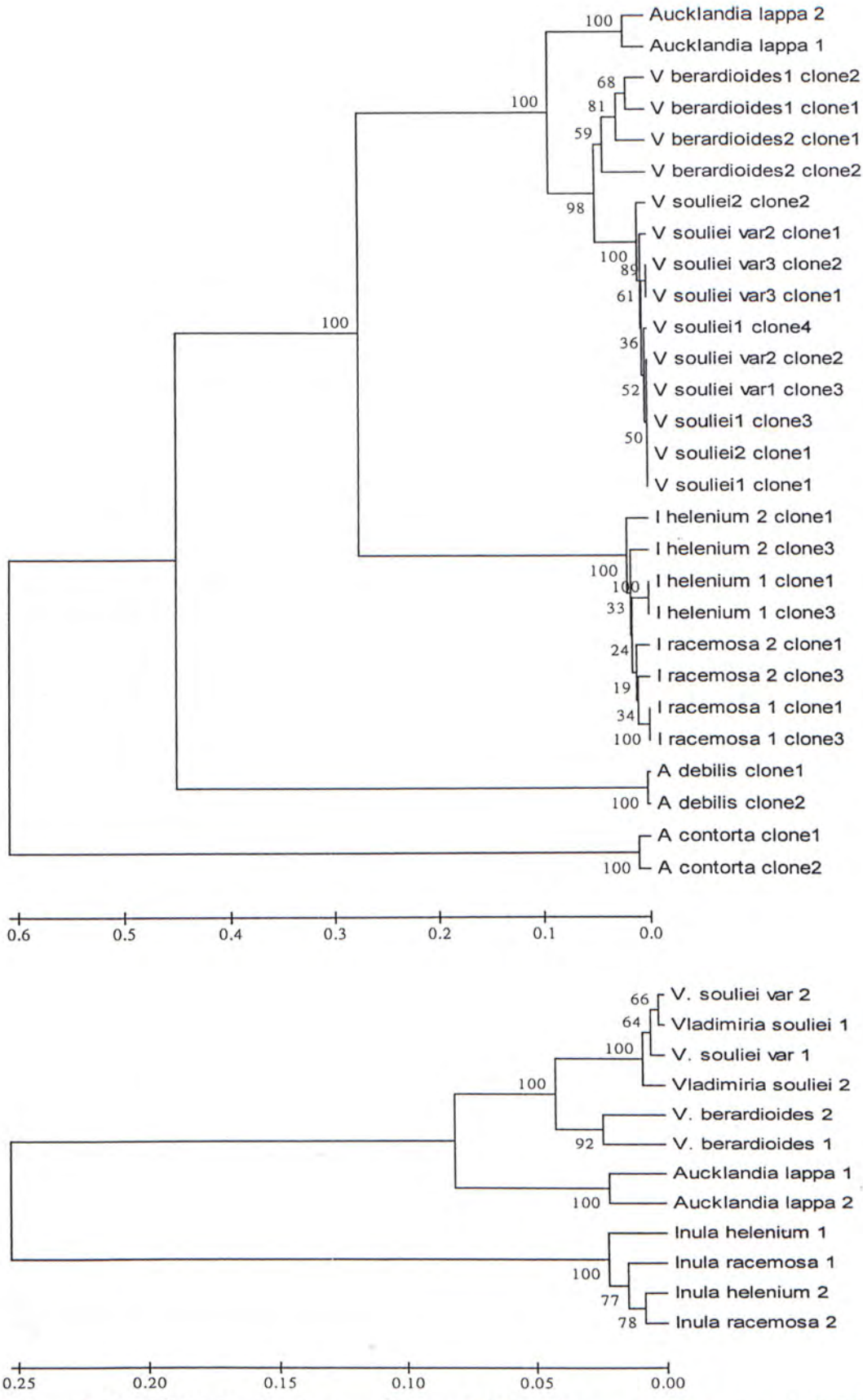

Figure 5-10. UPGMA Phylogram from nrDNA 5S spacer (Upper) and ITS2 (Lower) of *Aucklandia lappa* and related species. Sequence data is partially from Chen, 2004.

5.4.8 Contents of dehydrocostuslactone and costunolide

Dehydrocostuslactone and costunolide were chosen to be the chemical markers for assessment of *A. lappa* because of their pharmacological effects, their uniqueness and abundance in *A. lappa*. The average retention time of dehydrocostuslactone and costunolide were 55.60min and 45.89min respectively. Dehydrocostuslactone was also found in *V. soliei* but costunolide was not found in any other species. Although dehydrocostuslactone could be found in both *A. lappa* and *V. soliei*, their chemical profiles were very different as shown previously, and their use should not be interchanged.

Benefited from the automation in detection, peak areas can be obtained numerically by GC-MS. By analyzing a serial dilution of a purified chemical, a standardization curve can be plotted and used to find out the content of the chemical in a sample.

According to *Chinese Materia Medica* (Editorial Board of *Chinese Materia Medica,* State Administration of Traditional Chinese Medicine of People's Republic of China, 1998b) and other literatures (Singh *et al.*, 1992), the content of dehydrocostuslactone and costunolide in total essential oil of *A. lappa* should exceed 50%. Of the 27 *A. lappa* samples tested in this project, 13 (48%) samples are found to be below

standard.

5.4.9 Locality study

Samples of *Aucklandia lappa* were collected in the southern part of China. To reveal the relationship between the chemical content and growth location, locations of collection of sample are spotted in a map (Figure 5-11). Results showed that the quality of *A. lappa* was not related to the localities of the herb. Both up-to-standard and below-standard samples could be found in Sichuan, Chongqing, Guizhou and Yunnan. One sample was collected in Guangdong and found to be above standard, and two samples in Guangxi were found to be below standard. Results also revealed that some wild samples also met the requirement to be used in medicine.

Figure 5-11. Location of collection of *Aucklandia lappa*. Each spot represents a sample. Solid spots represent samples which are up to standard. Hollow spots represent samples which are below standard. Spots labeled with "W" refer to samples collected in the wild. Only the relative location (up to county level) is shown here.

5.5 Conclusion

GC-MS is a powerful technique and it can authenticate *A. lappa* and related species. The medicinal species *A. lappa* and *V. soliei* were distinguishable by hierarchical analysis. *I. helenium* was clustered with *I. racemosa*, meaning that the two species had similar chemical profiles. GC-MS could also assess the chemical components in *A. lappa* quantitatively. In this project, two chemical markers were chosen:

dehydrocostuslactone and costunolide. 48% samples of *A. lappa* were found not meeting the standard in which over 50% essential oil should be dehydrocostuslactone and costunolide.

Chapter 6. General Discussion

Two Chinese medicinal materials, Radix Aconiti and Radix Aucklandiae, were studied in this research project. In both types of materials, adulterants and below-standard samples were found on the market. This reveals that adulteration and sub-standard TCM products are common.

In order to ensure the efficacy and public safety of medicinal materials, authentication is necessary. In this study, both DNA and chemical techniques were applied, and they were proven successful to different extent in identifying adulterants and sub-standard samples.

In the first part, DNA sequencing was applied to distinguish between Pharmacopoeia-listed *Aconitum* species, namely *Aconitum carmichaeli* and *A. kusnezoffii*, and unlisted *Aconitum*. Sequence markers 5S spacer and *psb*A-*trn*H spacer were used. To further confirm the identities of the samples, genomic subtraction was applied to screen for differential markers in *Aconitum*.

In the second part, gas chromatography- mass spectrometry (GC-MS) was applied to

generate chemical profiles of *Aucklandia lappa* and its related medicinal species, namely *Vladimiria soliei*, *V. berardioides*, *Inula helenium* and *I. racemosa*. Also, standard chemicals from *A. lappa* were extracted and purified. By standardization in GC-MS analysis, their contents in each sample were determined.

6.1 DNA sequencing

DNA sequencing provides a definitive means for authentication. DNA being the genetic materials does not vary in different organs. Sequence information can still be extracted from minute amount of samples. Depending on the length of the markers, sequence information from short markers can still be obtained in case of DNA degradation. With automatic sequencers, a large number of samples can be analyzed in a short period of time.

In this project, sequence information of 5S spacer from nrDNA and *psb*A-*trn*H spacer from plastid genome was extracted from various *Aconitum* samples. By phylogram study with authentic samples, Pharmacopoeia-listed *Aconitum* was distinguished from the unlisted *Aconitum*. However, the two medicinal species, *Aconitum carmichaeli* and *A. kusnezoffii*, always clustered together. This result

means 5S spacer and *psb*A-*trn*H spacer are unable to discriminate the two.

6.2 Genomic subtraction

Common phylogenetic markers may not always be useful in distinguishing species. If the relationship between the genuine and adulterant species is too close, their marker sequences may not be variable enough for authentication. In this case, novel markers have to be found. Genomic subtraction is a way to screen for differential sequences between genomes, and the differential sequences may be used in species identification.

Since 5S spacer and *psb*A-*trn*H spacer could not be used in distinguishing the two Pharmacopoeia-listed species, genomic subtraction was applied. *Aconitum carmichaeli* ACfz2 and *A. kusnezoffii* AK2 were used in subtraction. A subtraction library was constructed and primers were designed to screen for markers in *Aconitum* samples. Three markers, SSH6, SSH15 and SSH45, were screened and phylograms from the three markers were also constructed. SSH15 was able to differentiate the Pharmacopoeia-listed *Aconitum* from the unlisted *Aconitum*. SSH6 could further discriminate *Aconitum carmichaeli* from *A. kusnezoffii*. On the other hand, SSH45,

together with 5S spacer, provided evidence for the hybridization during the evolution of *Aconitum*.

6.3 Future work on molecular authentication

Authentication by DNA sequencing has become popular in recent years. Several medicinal species were successfully authenticated by this method (Lau *et al.*, 2000; Ngan *et al.*, 1999; Wong *et al.*, 2004). There is much sequence information of different species published in GenBank. However, due to a wide variety of Chinese medicinal materials, there is still a long way to extend this technique to most of the medicinal species. As the effectiveness of DNA authentication has been confirmed, its use should widely be explored in other medicinal species. On those species which cannot be discriminated by traditional phylogenetic markers, the new method of screening novel markers, genomic subtraction, should be promptly applied.

6.4 Future work on authentication of *Aconitum*

Aconitum, which consists of around 400 species, is a large genus. It is essential to confirm further the differential ability of the sequence markers, including 5S spacer,

*psb*A-*trn*H spacer, SSH6 and SSH15. Literatures suggested that the Pharmacopoeia-listed *Aconitum* (*A. carmichaeli* and *A. kusnezoffii* in Ser. Inflata) was evolved from *A. volubile* (Ser. Volubilia) (Luo *et al.*, 2005; Kita and Ito, 2000). To elucidate the complicated relationship among Ser. Volubilia and Ser. Inflata, samples from the two series should be collected for a more detailed phylogenetic study which may also facilitate the authentication of medicinal *Aconitum*.

On the other hand, there are still many clones in the subtraction library unexplored. In the future, more markers can be screened by using the methods described in this project.

6.5 Gas chromatography- mass spectrometry

By combining two powerful analytical techniques, gas chromatography and mass spectrometry, GC-MS provides a rapid method to analyze a mixture of chemicals even from minute amount of sample. The resolution of gas chromatography is so high that even chemicals with similar structures can still be separated. With robotic machinery, GC-MS can work without human operation.

In this project, the essential oil contents from the root part of *Aucklandia lappa*, *Vladimiria soliei*, *V. berardioides*, *Inula helenium* and *I. racemosa* were analyzed by GC-MS to generate a chemical profile for each sample. By chemometric analysis, the relationship among the samples was revealed. Results showed that the chemical content varied among different species and their medicinal use should not be interchanged.

6.6 Future work on authentication by GC-MS

There is a limitation in using GC-MS. As GC-MS requires separation in gas phase, only chemicals which are volatile (at least volatile at the highest oven temperature) and not heat-labile can be analyzed by GC-MS.

Despite its limitation, GC-MS can still be widely applied in authentication of Chinese medicinal materials which contain volatile components providing pharmacological effects. The use of GC-MS should be explored in authentication of other medicinal materials.

GC-MS can also be used to analyze the body fluids so as to find out what CMM(s)

is/are taken by patients. This is particularly useful in detecting chemicals in patients who are intoxicated by over-dosage or mis-use of CMM(s). GC-MS can provide a fast analysis which ensures that a quick and proper decision of a suitable treatment can be made.

6.7 Future work on authentication of *Aucklandia lappa* and related species

Authentication of *Aucklandia lappa* and related species by GC-MS provides quality assessment of each sample. However, not all samples meet the standard. To ensure efficacy of *A. lappa*, GC-MS should be introduced as a regular analysis for quality control.

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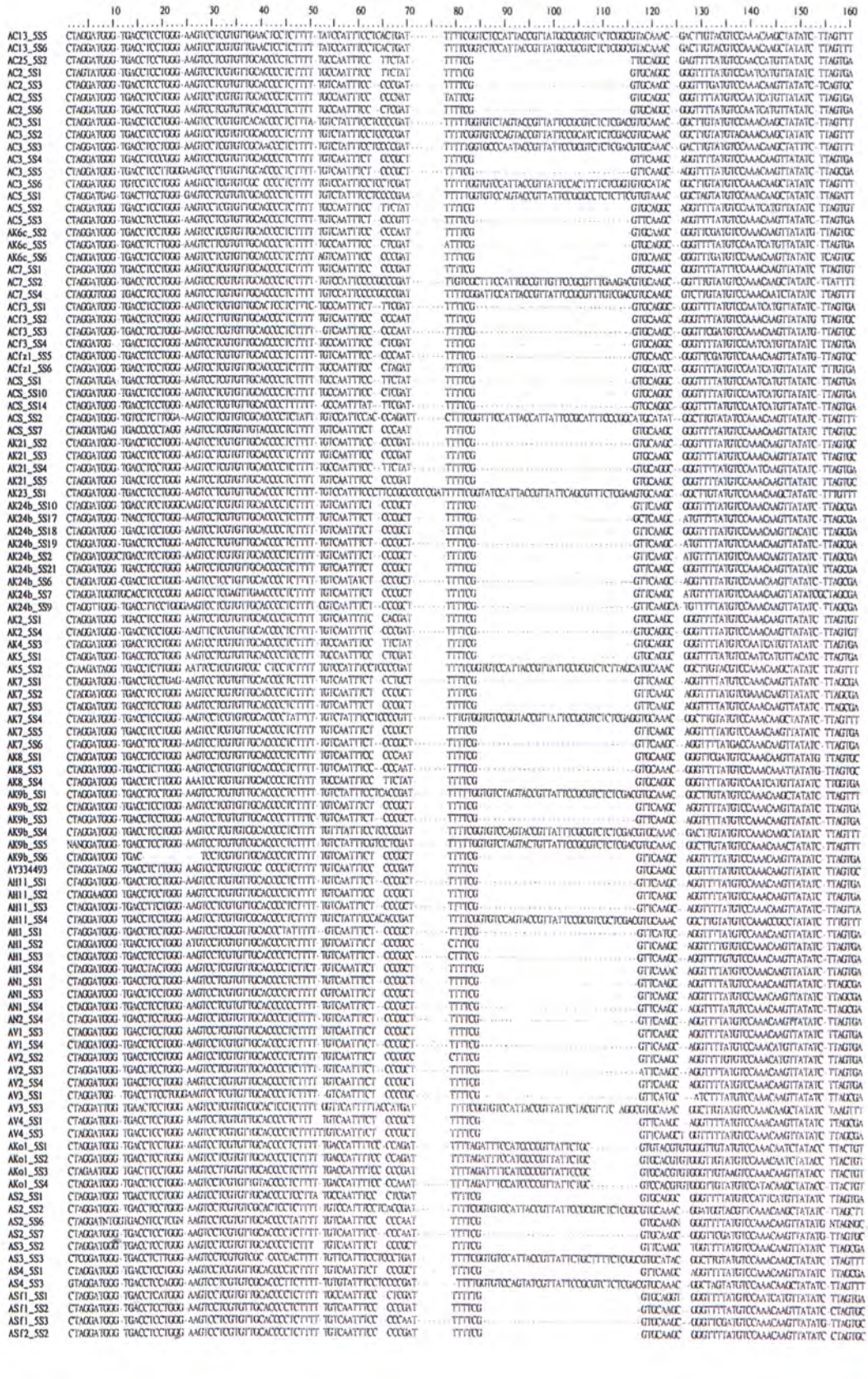
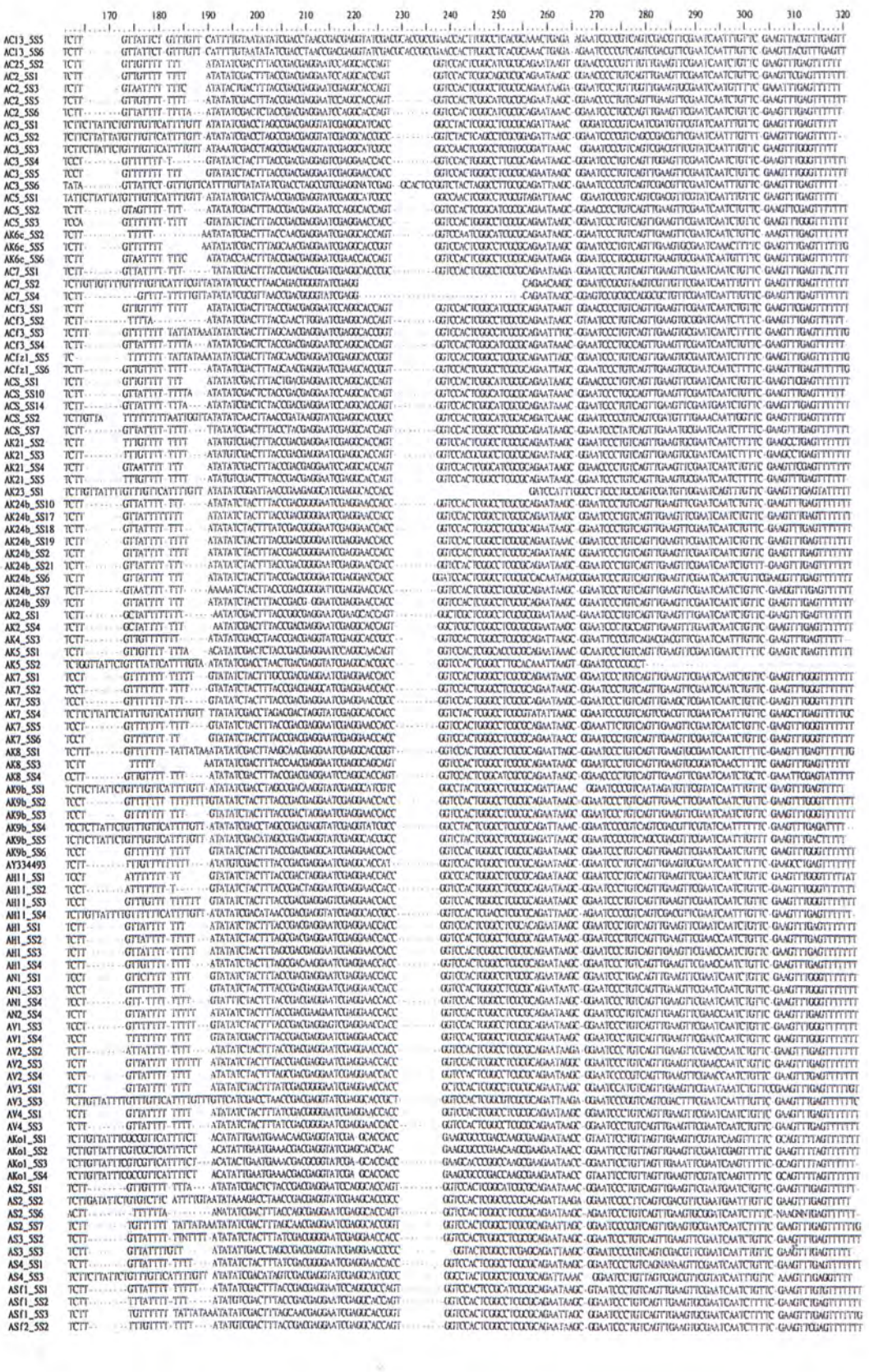
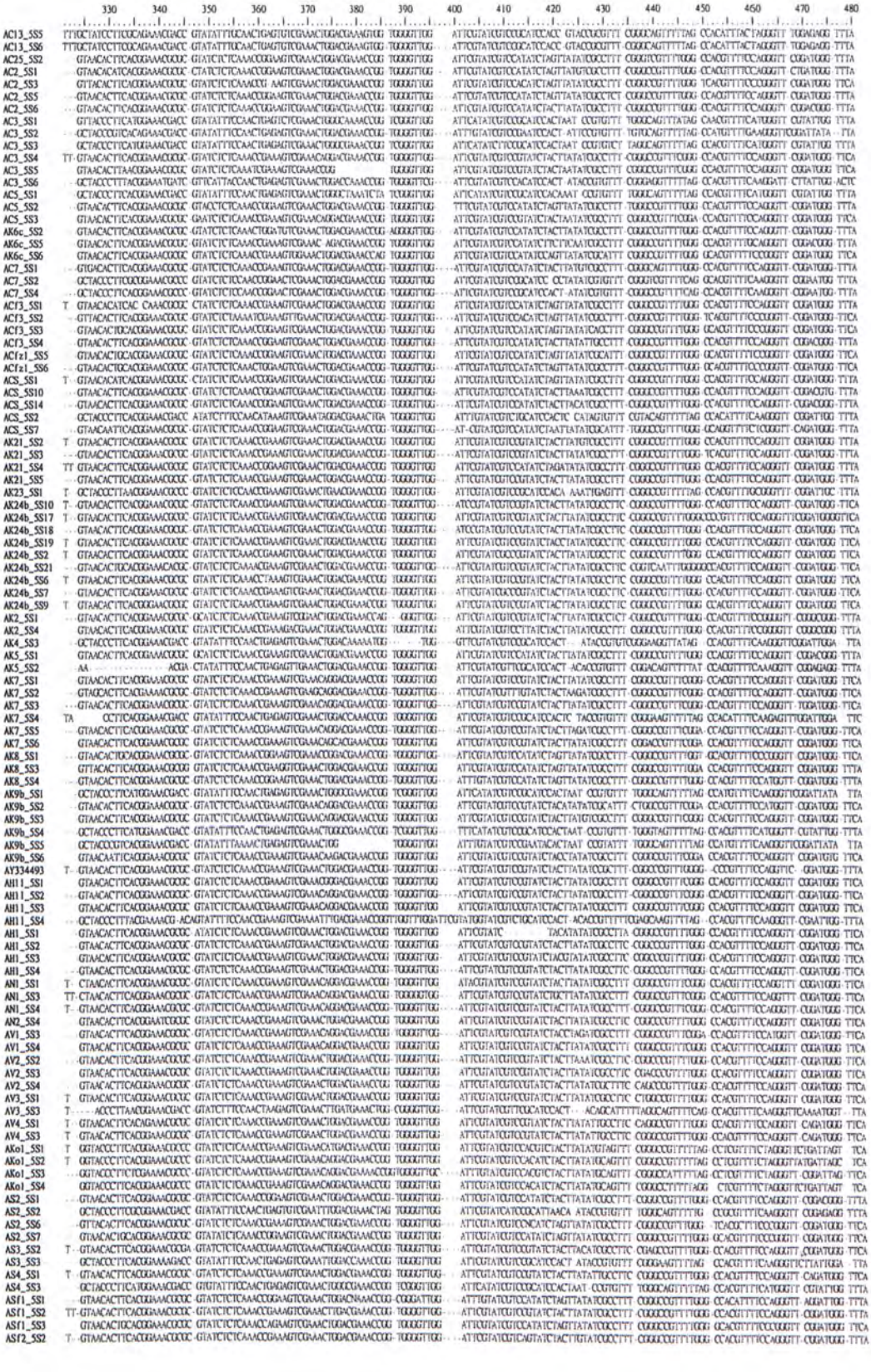
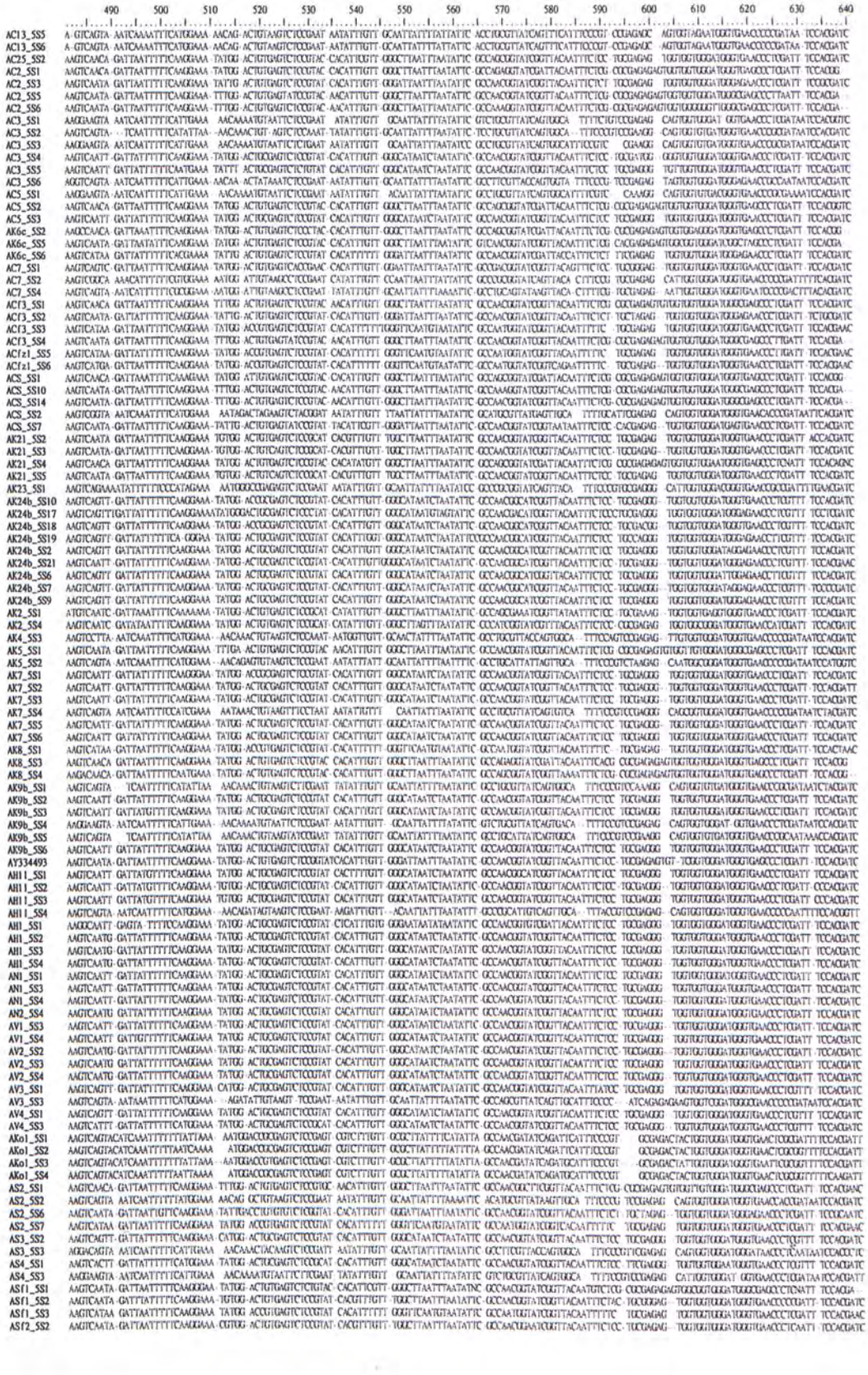
Appendix A. Sequence Alignment of 5S Spacer from *Aconitum* Species





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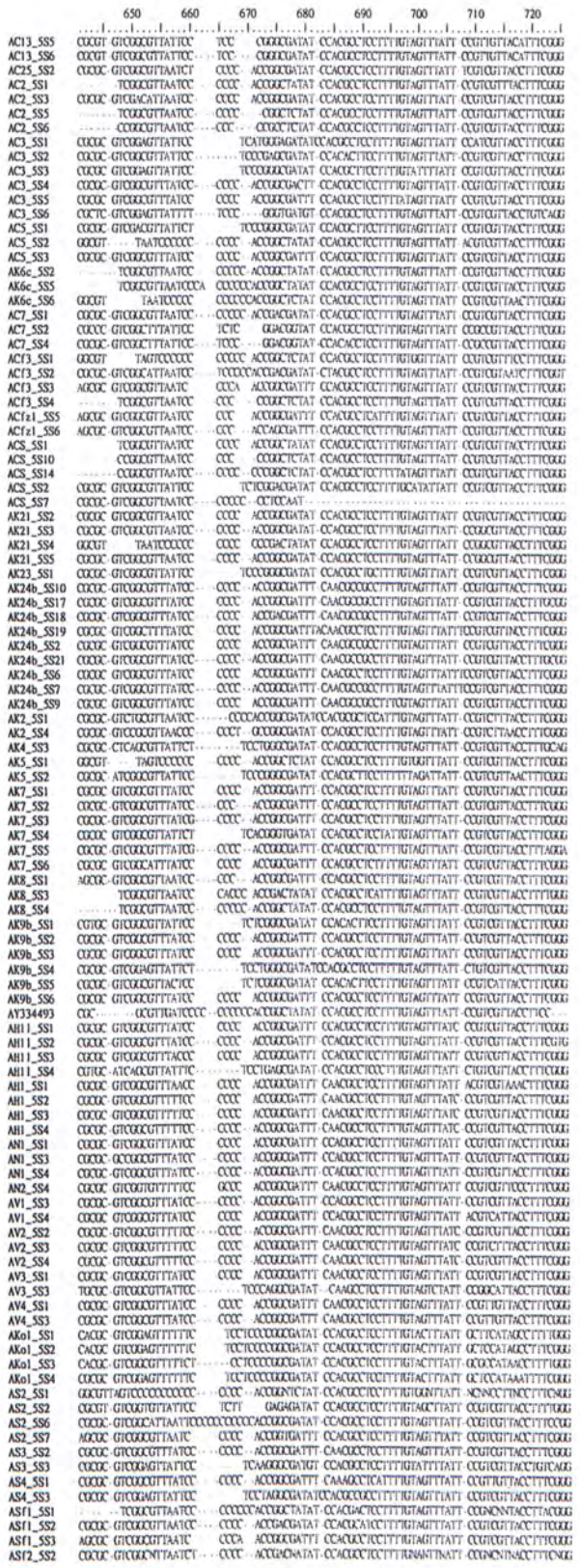
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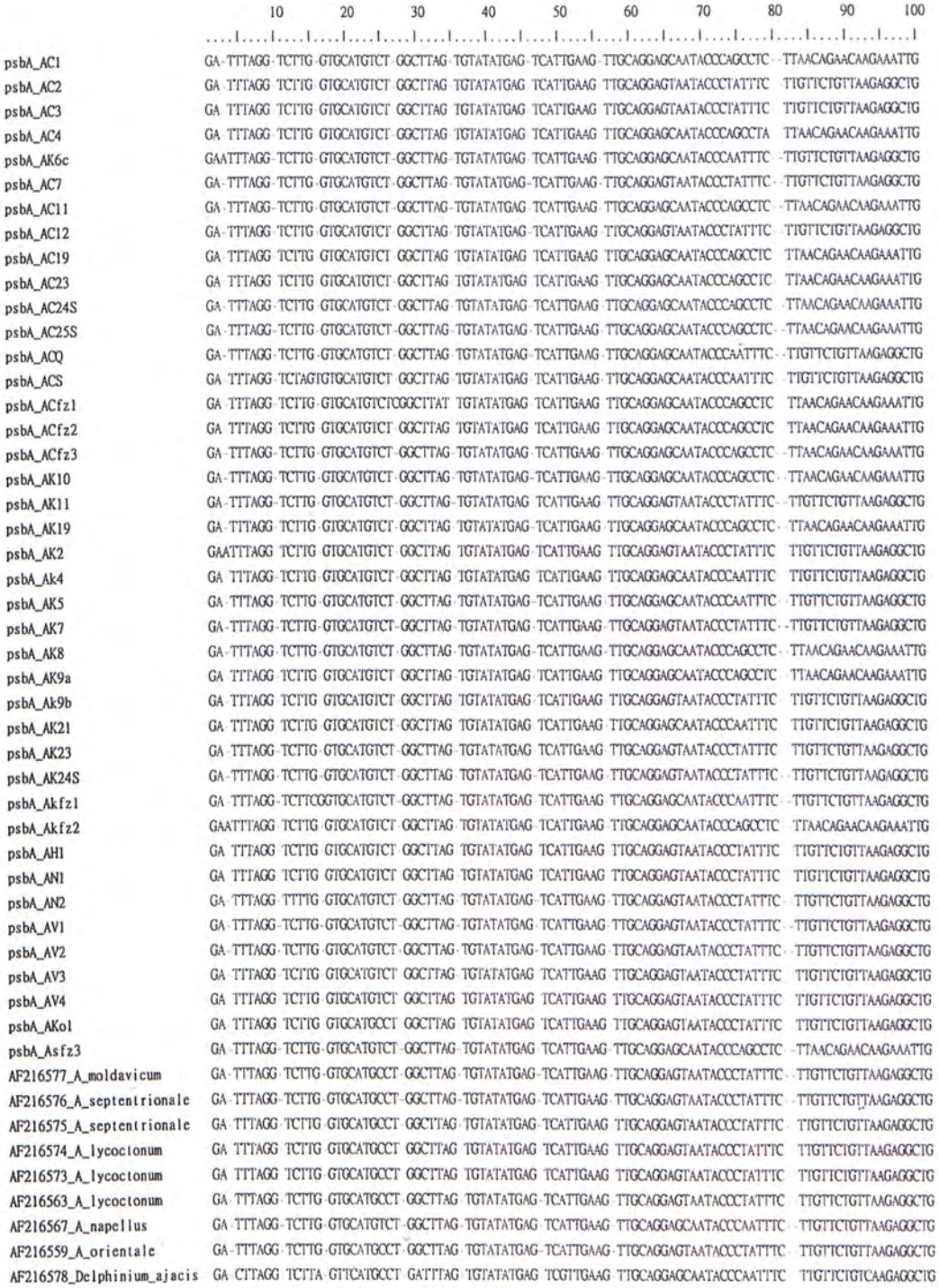
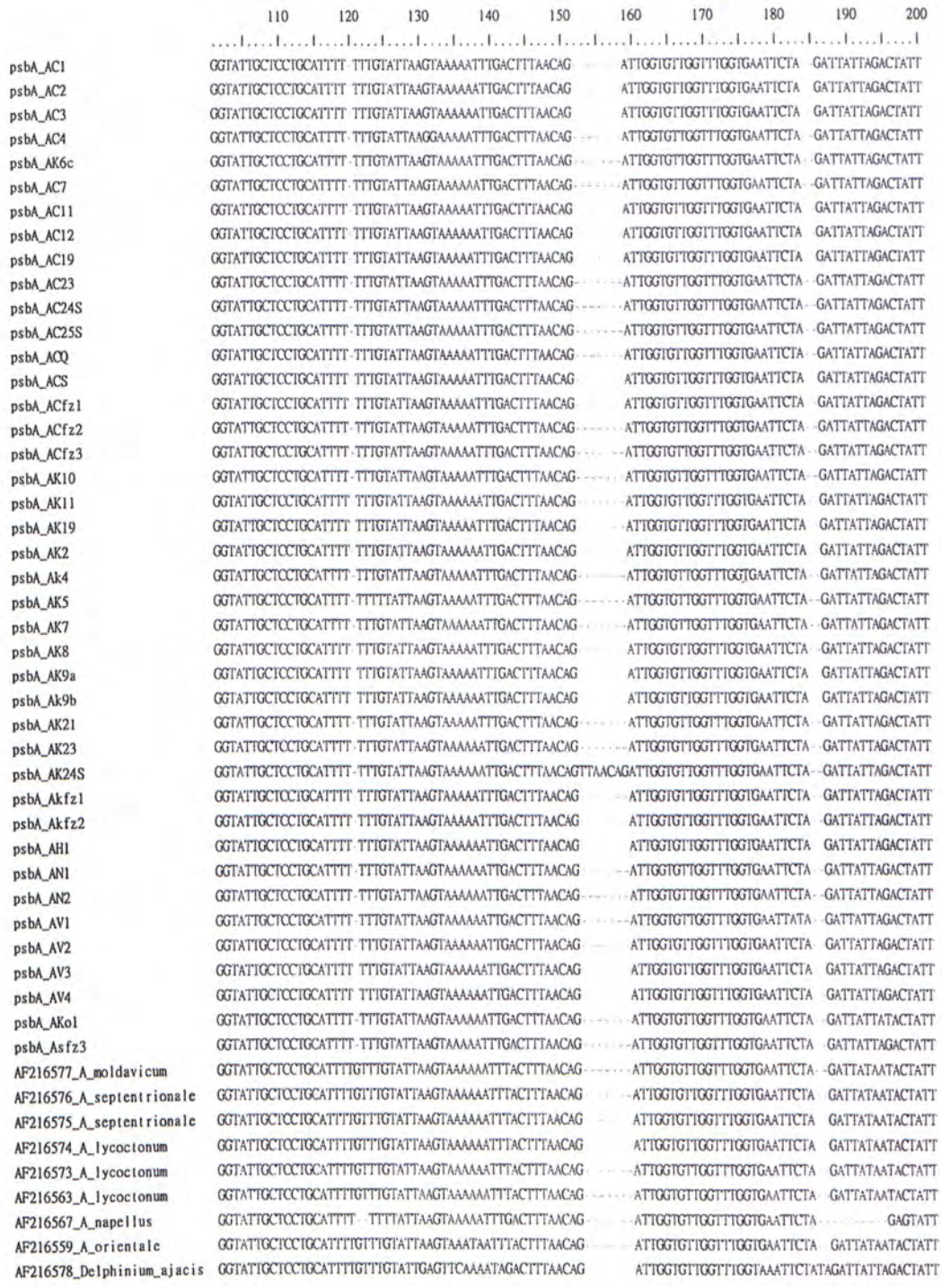
Appendix B. Sequence Alignment of *psb*A-*trn*H Spacer from *Aconitum* Species

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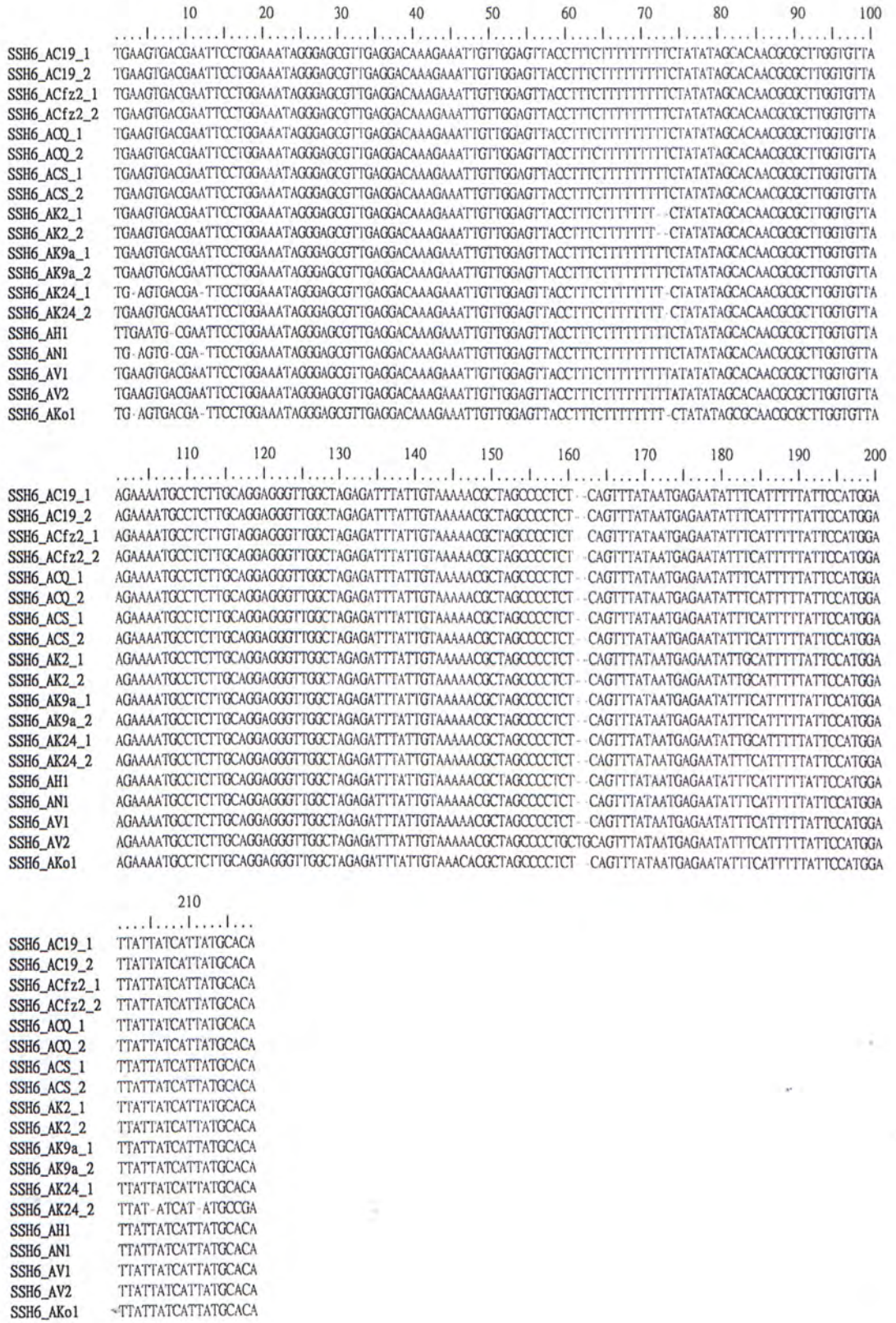
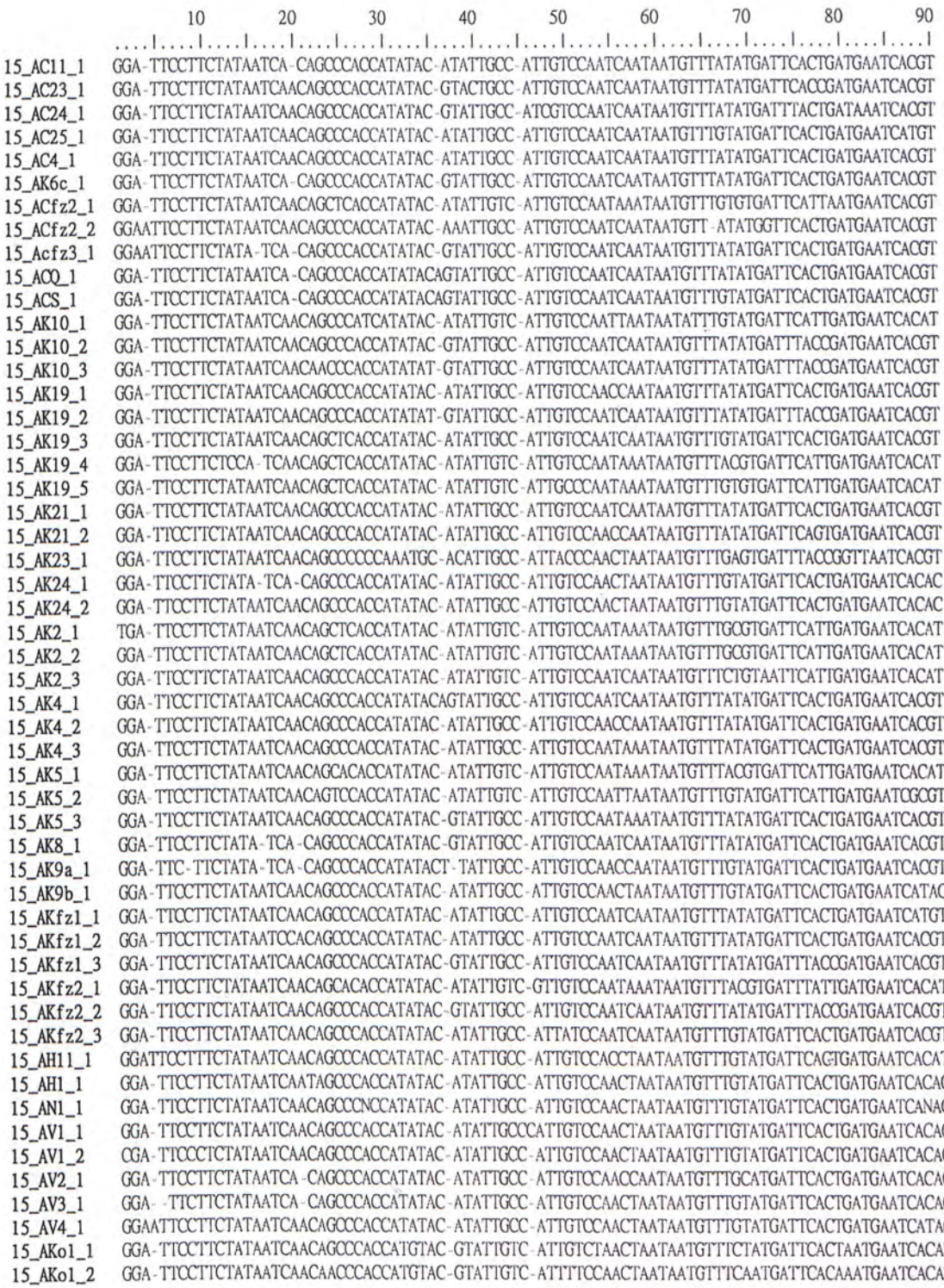
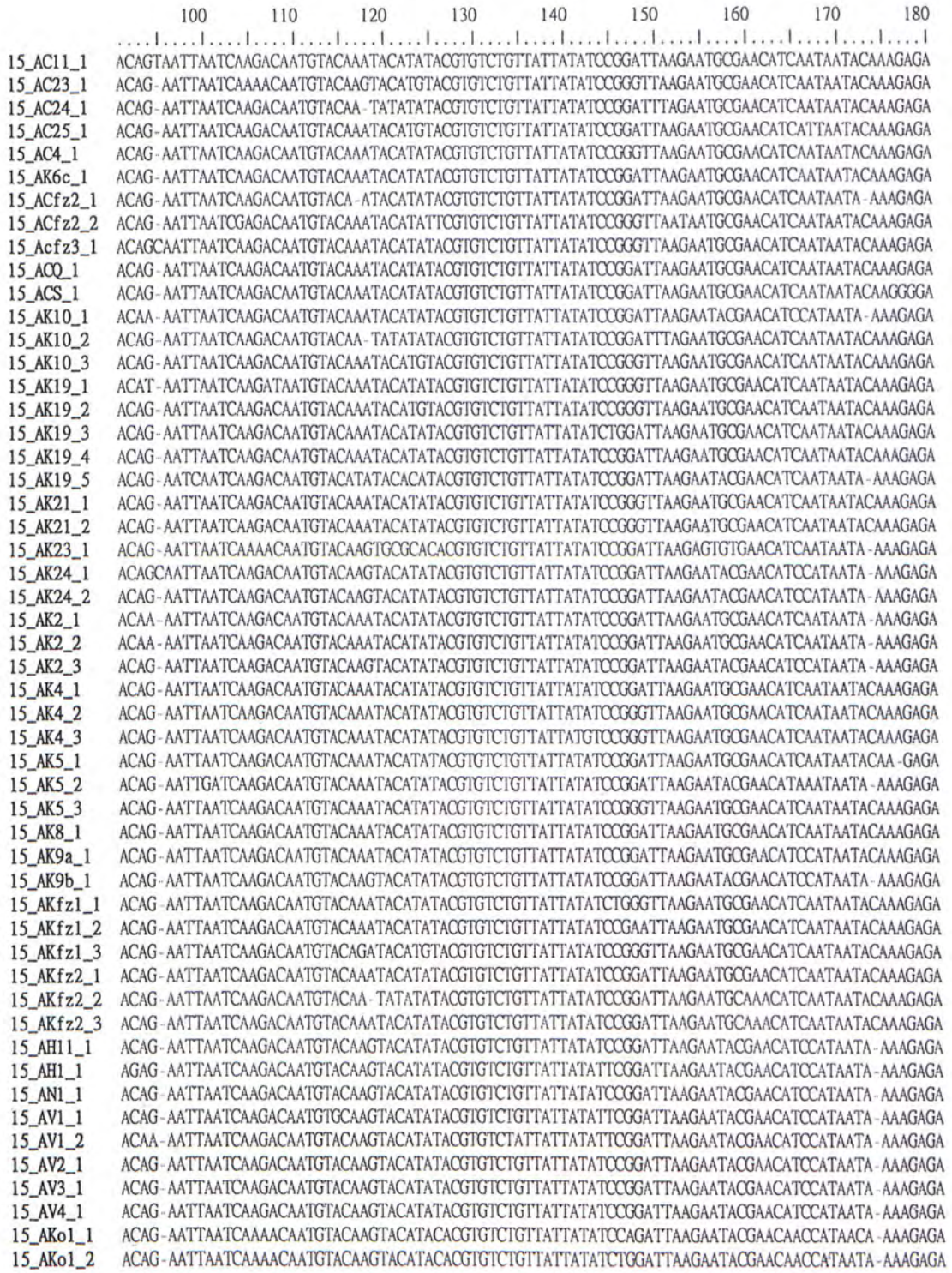
Appendix D. Sequence Alignment of SSH6 from *Aconitum* Species



Appendix E. Sequence Alignment of SSH15 from *Aconitum* Species

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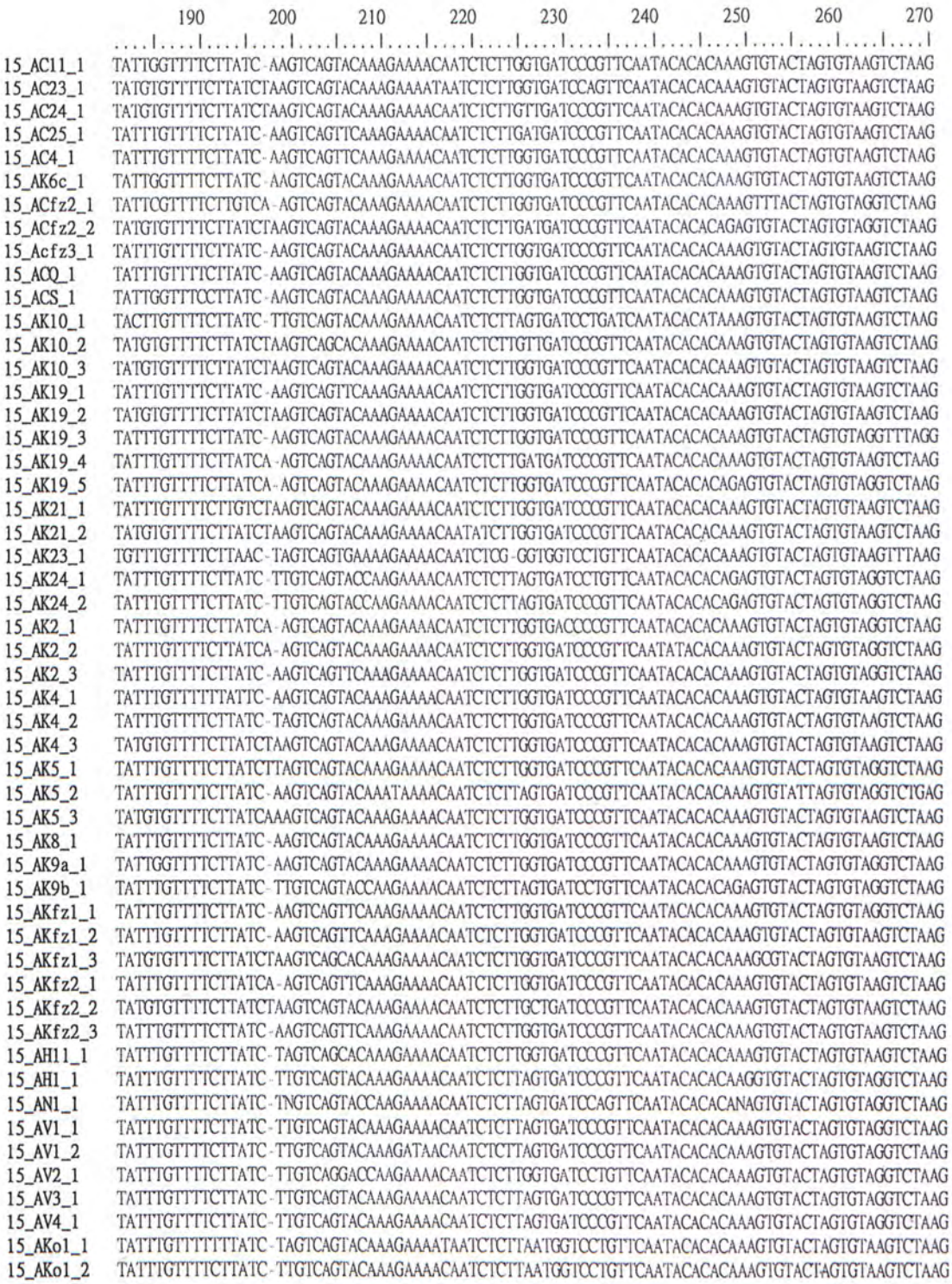
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15_AC11_1        TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AC23_1        TCAAGATAGACTAATACTGACTGACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AC24_1        TCAAGATAGACTAATACCGACTGACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATC-ACTT-ACATT
15_AC25_1        TCAAGATAGACTAATGCTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AC4_1         TCAAGATAGACTAATACTGACTCACACTACAACT-GTATGCAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK6c_1        TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_ACfz2_1       TCAAGACAGACTAATACCGACTTACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTT-CAGTTGTGAACAGT-ACTT-ACATT
15_ACfz2_2       TCAAGACAGACTAATACCGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTT-CAGTTGTGAACATT-ACTT-ACATT
15_Acfz3_1       TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATC-ACTT-ACATT
15_ACQ_1         TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_ACS_1         TCAAGACAGACTAATACNGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK10_1        TCAAGACAGACTAATACCGACTTACCCTACAACT-GTAC-CAATAGTTTATCC-AAATCCATTT-AAGTTGTGCACCTT-ACTT-ACATT
15_AK10_2        TCAAGATAGACTAATACCAACTGACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATC-ACTT-ACATT
15_AK10_3        TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK19_1        TCAAGATAGACTAATACTGATTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK19_2        TCAAGATAGACTAATACTGACTGACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK19_3        TCAAGACAGACTAATACCGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATT--CAGTTGTTAACCGT-ACTT-ACATT
15_AK19_4        TCAAGACAGACTAATACCGACTCACACTACATCT-GTAT-CAATGGTTTATCC-AAATCCATTT-CATTTGTGAACCGT-ACTT-ACATT
15_AK19_5        TCAAGACAGACTAATACCGACTCACACTACAACT-GTAT-TAATGGTTTATCC-AAATCCATTT-CAGTTGTGAACTGT-ACTT-ACATT
15_AK21_1        TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTCATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK21_2        TCAAGATAGACTAATACTGACTGACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK23_1        TCAAGACAGACTAATACCAACTCCCACTACAACCTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGACCCTT-ACTT-ACATT
15_AK24_1        TCAAGACAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCCCAAATCCATTT-AAGTTGTGAACTGT-TCTT-ACATT
15_AK24_2        TCAAGACAGACTAATACCAACTCACACTACAAGCTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACTGT-TCTT-ACATT
15_AK2_1         TCAAGACAGACTAATACCGACTTACACTACAACT-ATAT-CAATGGTTTATCC-AAATCCATTC-AAGTTGTGAACATT-ACTTTACATT
15_AK2_2         TCAAGACAGACTAATACCGACTTACACTACAACT-ATAT-CAATGGTTTATCC-AAATCCATTC-AAGTTGTGAACATT-ACTTTACATT
15_AK2_3         TCAAGACAGACTAATACTGACTCACACTACAACTTGTAT-CAATGGTTTATCC-AAATCCATTC-AAGTTGTGAACATTTACTT-ACATT
15_AK4_1         TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK4_2         TCAAGATAGACTAATACCGACTTACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTT-CAGTTGTGAACTGT-ACTT-ACATT
15_AK4_3         TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK5_1         TCAAGACAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CATTTGTGAACACT-ACTTTACATT
15_AK5_2         TCAAGACAGACTAATACCGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTC-AAGTTGTGAACATT-ACTT-ACATT
15_AK5_3         TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ATTT-ACATT
15_AK8_1         TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AK9a_1        TCAAGACAGACTAATACCGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTATCAGTTGTGAACAGT-ACTT-ACATT
15_AK9b_1        TCAAGACAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACTGT-TCTT-ACATT
15_AKfz1_1       TCAAGACAGACTAATACCGACTCATACTACAACT-GTAA-CAATGGTTTATCC-ATATCCATTT-CAGTTGTGAACCTT-ACTT-ACACT
15_AKfz1_2       TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AKfz1_3       TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATT-ACTT-ACATT
15_AKfz2_1       TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAACGGTTTATCC-AAATCCAT-A-CAGTTGTGAACATT-ACTT-ACATT
15_AKfz2_2       TCAAGATAGACTAATACCGACTGACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAACATC-ACTT-ACATT
15_AKfz2_3       TCAAGATAGACTAATACTGACTCACACTACAACT-GTAT-CAATGGTTTATCC-AAATCCATTA-CAGTTGTGAATATT-ACTT-ACATT
15_AH11_1        TCAAGACAGACTAATACCGACTCACACTACAACT-GTAC-CAATGGTTTATCC-AAATCCATTTAAGGTTGTGAACCTT-ACTT-ACATT
15_AH1_1         TCAAGACAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACTGT-TCTT-ACATT
15_AN1_1         TCAAGACAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACTGT-TCTT-ACATT
15_AV1_1         TCAAGATAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACAGT-TCAT-ACATT
15_AV1_2         TCAAGATAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAATTGT-TCTT-ACATT
15_AV2_1         TCAAGACAGACTAATACCGACTCACACTACAA-CTGTAC-CAATGGTTTATCCAAATNCCATTT-AAGTTGTGAACTGT-ACTT-ACATT
15_AV3_1         TCAAGACAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACTGT-TCTT-ACATT
15_AV4_1         TCAAGACAGACTAATACCAACTCACACTACAA-CTGTAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACTGT-TCTT-ACATT
15_AKo1_1        TCAAGACAAACTCATACCAACTCACACTACAATT-GTAC-CAATGGTTTATCT-AAATCCATTT-AAGTTGTGAACTTT-ACTT-ACATT
15_AKo1_2        TCAAGACAGACTCATACCAACTCACACTACAACT-ATAC-CAATGGTTTATCC-AAATCCATTT-AAGTTGTGAACCAT-ACTT-ACATT
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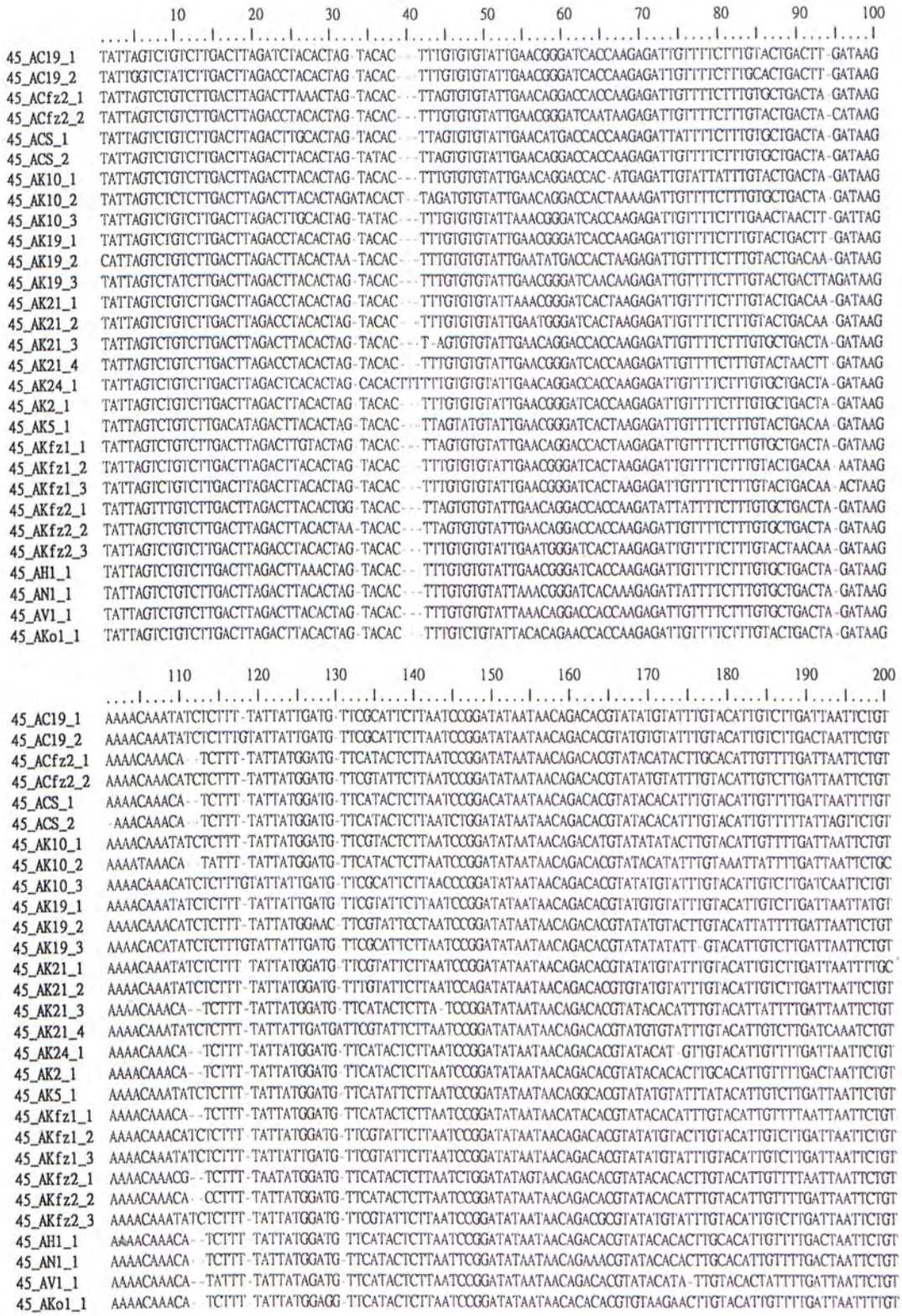
                 370        380        390        400        410
                 ....|....|....|....|....|....|....|....|....|...
15_AC11_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AC23_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AC24_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AC25_1        CTA-CAAGGAACT-GATAAC-ATGATCTTCA-GTGTGACACAACACA-CCATG
15_AC4_1         CTA-CAAGGAACT-GATAAC-ATGATCTTCATGTGTGACACAACACA-CCATG
15_AK6c_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_ACfz2_1       CTA-CAAGGAACTTGATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_ACfz2_2       CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_Acfz3_1       CTAGCAAGGAACT-GATAAC-ATGATCTT-CAGTG-GACACAACGCT-CCAGA
15_ACQ_1         CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCTGT
15_ACS_1         CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTG-GACACAACACA-CCATG
15_AK10_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACTACACA-CCATG
15_AK10_2        CTA-CAAGGAACT-GATAAC-ATGATCTT-CTGTGTGACACAACACA-CCATG
15_AK10_3        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK19_1        CTA-CAAGGAACT-GATAAC-ATGATCTTCA-GTGTGACACAACACA-CCATG
15_AK19_2        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK19_3        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCTTG
15_AK19_4        CCA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK19_5        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK21_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK21_2        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK23_1        CTAGCAAGGAAGCTGATAAC-ATGATCTT-CTGTGATCTACCACACAGCCATG
15_AK24_1        CTA-CAAGGAACT-GATCAC-ATGATCTTC-AGTGTGACACAACACA-CCATG
15_AK24_2        CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACTCT-CCAGA
15_AK2_1         CTA-TAGGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK2_2         TTA-CAGGGAACT-CATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK2_3         CTA-CAAGGGACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK4_1         CTA-CAAGGAACT-GATAAC-ATGATCTTTCAGTGTGACACAACACA-CCATG
15_AK4_2         CTA-CAAGGAACT-GATAAC-ATGATCTT-CTGTGTGACACAACACA-CCATG
15_AK4_3         CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK5_1         CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK5_2         CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AK5_3         CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAGCACA-CCATG
15_AK8_1         CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTG-GACACAACACA-CCATG
15_AK9a_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTG-GACACAACACA-CCAGA
15_AK9b_1        CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACACC-CCATG
15_AKfz1_1       CTA-CAAGGAACT-GATAAC-ATGATCTT-CCGTGTGACACAACACA-CCATG
15_AKfz1_2       CTA-CAAGGAACT-GATAAC-ATGATCTTCA-GTGTGACACAACACA-CCATG
15_AKfz1_3       CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AKfz2_1       CTA-CAAGGAACT-GATAAC-ATGATCTT-CAGTGTGACACAACACA-CCATG
15_AKfz2_2       CTA-CAAGGAACT-GATAAC-ATGATCTT-CTGTGTGACACAACACA-CCATG
15_AKfz2_3       CTA-CAAGGAACT-GATAAC-ATGATCTTCA-GTGTGACACAACATA-CCATG
15_AH11_1        CTA-CAAGGGACT-GATAACCATGATCTTCATGTGTGACACAACACA-CCCAG
15_AH1_1         CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACACA-CCATG
15_AN1_1         CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACACA-CCATG
15_AV1_1         CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACACA-CCATG
15_AV1_2         CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACACA-CCATG
15_AV2_1         CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACACA-CCATG
15_AV3_1         CTA-CAAGGAACT-GATAAC-ATGATCTTCCAGTGTGACACAACACA-CC-CA
15_AV4_1         CTA-CAAGGAACT-GATAAC-ATGATCTTC-AGTGTGACACAACACA-CCATG
15_AKo1_1        CTA-CAAGGAACT-GATAAC-ATGATCTT-CTGTGTGACACAACACA-CCATG
15_AKo1_2        CTA-CAAGGAACT-GATAAC-ATGATCTT-CTGTGTGACACAACACA-CCATG
```

Appendix F. Sequence Alignment of SSH45 from *Aconitum* Species



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                 210        220        230        240        250        260        270        280        290        300
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
45_AC19_1        ATGTGATTCGTCAATGAATCACA-CAAACATTATTTATTGGACAATGACAATACGTATATGGTGAGCTGTTGATTATAGAA-GGAATCCATGTACCGATA
45_AC19_2        ATGTGATTCATCAATGAATTACG-GAAACATTATTGATTGGACAATGACAATATGTATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_ACfz2_1       AAGTGATTCATCAGTGAATCATG-TAAACATTATTAATTGGACAATGACAATGTGCATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_ACfz2_2       ATGTGATTCATCAATGAATCATA-CAAACATTATTAATTGGACAATGACAATATGTATATGGTGGGCTGTTGATTATAGGA-GGAATCCGTGTCCCGATA
45_ACS_1         AAGTGATTCATCAGTGAATCATG-CAAACATTATTAGTTGGACAATGACAATGTGCATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_ACS_2         AAGTGATTCATCAGTGAATCGTG-TAAATATTATTAGTTGGACAATGACAATGTGCATATGGTGGGCTGTTGATTATAAAA-GGAATCCATGTCCCGATA
45_AK10_1        ATGTGATTCATCAATGAATCATT-AAAACATTATTAGTTGGACAATGGCAATATGTATATGGTGGGCTGTTGATTATAAAA-GGAATATATGTCCCGATA
45_AK10_2        AAGTGATTCATCAGTGAATCATG-TAAACATTATTAGTTGGACAATGACAATGTGCATATGGTGGGTTGTTGATTATAAAAAGGAATCCATGTCCCGATA
45_AK10_3        ACGTGATTCATCAGTGAATCATA-TAAACATTATTGATTGGACAATGACAATATGTATATGGTGGACTATTGATTATAGAA-GGAATCCATGTCCCAATA
45_AK19_1        ATGTGATTCATCAATGAATCACA-CAAACATTATTTATTGGACAATGACAATATGTATATGGTGAGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AK19_2        AAGTGATTCATCAGTGAATCATG-TAAACATTATTAGTTGGACAATAATAATATGTATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AK19_3        ACGTGATTCATCAGTGAATCATA-TAAACATTATTGATTGGACAATGGCAATACATATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AK21_1        ATGTGATTCATCAATGAATCATA-CAAACATTATTAATTGGACAATGACAATATGTATATGGTGGGTTATTGATTATAGAA-GGAATCCATGTCCCGATA
45_AK21_2        ATGTGATTCATCAATGAATCATA-CAAACATTATTAATTGGATAATGACAATATGTATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AK21_3        AAGTGATTCATCAGTGAATCATG-TAAATATTATTAGTTGGACAATGACAATGTGCATATGGTGGGTTGTTGATTATAAAA-GG-ATCCATGTCCCGATA
45_AK21_4        ATGTGATTCATCAGTGAATCACATCAAACATTATTTACTGGACAATGACAATACGTATATGGTGAGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AK24_1        ACGTGATTCATCAGTGAATCATA-TAAACATTATTAGTTGGATAATGGCAACGTGTATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AK2_1         AAGTGATTCATCAGTGAATCATG-TAAACATTATTAATTGGACAATGACAATGTGCATATGGTGGGTTGTTGATTATAGAA-GGAATCCATGTCTCGATA
45_AK5_1         ACGTGATTCATCAGTAAATCACA-CAAACATTATTAATTGGACAATGACAATATATATATGGTGGGCTATTGATTATAGAA-GGAATCCATGTCCCGATA
45_AKfz1_1       AAGTGATTCATCAGTGAATCATG-TAAACATTATTAGTTGGATAATGACAATGTGCATATTGTGGGCTGTTGATTATAAAA-GGAATCCATGTCTCGATA
45_AKfz1_2       GTGTGATTCATCAGTGAATCATA-CAAACATTATTAGTTGGACAATGACAATATGTATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AKfz1_3       ATGTGATTCATCAATGAATCATA-CAAACACTATTAATTGGACAATGACAATATGTATATGGTGGGTTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AKfz2_1       AAGTAATTCATCTGTGAATCATG-TAAACATTATTAGTTGGAAAATGACAATGTGCATATGGTGGGCTGTTGATTATAAAA-GGAATCCATGTCCCGAT-
45_AKfz2_2       AAGTGATTCATCAGTGAATCATG-TTAACATTATTAGTTGGACAATGACAATGTGCATATGGTGGGCTGTTGATTATAAAA-GGAATCCATGTCCCGATA
45_AKfz2_3       GTGTGATTTATCAGTGAATCATA-CAAACATTATTAGTTGGACAATGACAATATGTATATGGTGGACTGTTGATCATAGAA-GGAATCCATGTCCCAATA
45_AH1_1         AAGTGATTCATCAGTGAATCATG-TAAACATTATTAAA-GGACAATGACAATGTGCATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AN1_1         AAGTGATTCATCAGTGAATCATG-TAAACATTATTAATTGGACAATGACAATGTGCATATGGTGGGTTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AV1_1         ACGTGATTCATCAGTGAATCATA-TAAACATTATTAGTTGGATAATGGCAACGTGTATATGGTGGGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA
45_AKo1_1        ATGTGATTCATTTGTGAATCATA-GAAACATTATTAGTTGGACAATGACAATAAATATATGGTGTGCTGTTGATTATAGAA-GGAATCCATGTCCCGATA

                 310        320        330        340        350        360        370
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|..
45_AC19_1        TAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCGTGTAAACCTTGCAGGTGGA-TTTGATCC
45_AC19_2        TAGTTT-GGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGTTTTGTCGTGTAAACCCTGCAGGTGGATTATGATCC
45_ACfz2_1       TGGTTTTGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGTTTGTCATGTAAACCTTGCAGGTGGA-TTTGATCC
45_ACfz2_2       TAGGTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-ATTGATCC
45_ACS_1         CAGTTTCGGG-TTGATGATGTCCTCTTAAGGAGCTCATA-GGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCT
45_ACS_2         CGGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK10_1        CAGTTTCGGG-TTGATGATGTCCTCTTGAGGAACTCTTAAGGTTTGTCATGTAAACCCTGCAGGTGGA-TTTAATTC
45_AK10_2        CGGTTTTGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK10_3        CAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTTATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK19_1        TAGTTTCGGG-ATGATGATGTCCTCTTGAGGAGCTCATAAGGATTATTGTGTAAACCCTGCTGGTGGA-TTTGATCC
45_AK19_2        CAGTTTCGGG-TTGATAATGTCCTCTTGAGGAGCTCATAAGGATTGCCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK19_3        TGATTTCGGGATTGATGATATCCTCTTGAGGAGCTCATAAGGATTGTCGTGCAAACCCTGCAGGTGGATTATGATCC
45_AK21_1        TAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGTTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK21_2        TAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCGTGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK21_3        CGATTTCGGG-TTAATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK21_4        TGGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCGTGTAAACCCTGCAGGTGGA-TTTGGTCC
45_AK24_1        CGGTTTCGGG-GTGATGATGTCCTCTTGAGGAGCTCATAAGGTTTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK2_1         CGGTTTCGGG-TTGATAATGTCCTCTTGAGGAGCTCATAAGGTTTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AK5_1         CAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AKfz1_1       CAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGTTTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AKfz1_2       TAGTTTCGGG-TTGATGATGTCCTATTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AKfz1_3       TAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCGTGTAAACCCTGCAGGTGGA-TTTGATCC
45_AKfz2_1       ----------TGATGATGTCCTCTTGAGGAGCTTATAAGGTTTGTCATGTAAACCCTGCAGGTGTA-TTTCATCC
45_AKfz2_2       CAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AKfz2_3       CAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGATTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AH1_1         TGGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGTTTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AN1_1         CGGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAAGGTTTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AV1_1         CGGTTTCGGG-TTGATGATGTCCTCTTGAGGAGTTCATAAGGTTTGTCATGTAAACCCTGCAGGTGGA-TTTGATCC
45_AKo1_1        CAGTTTCGGG-TTGATGATGTCCTCTTGAGGAGCTCATAATGATTGTCATGTAAACCCTGTAGGTGGATTATGATCC
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Appendix G. Gas Chromatograms of Essential Oil Extracts of *Aucklandia lappa* and Related Species

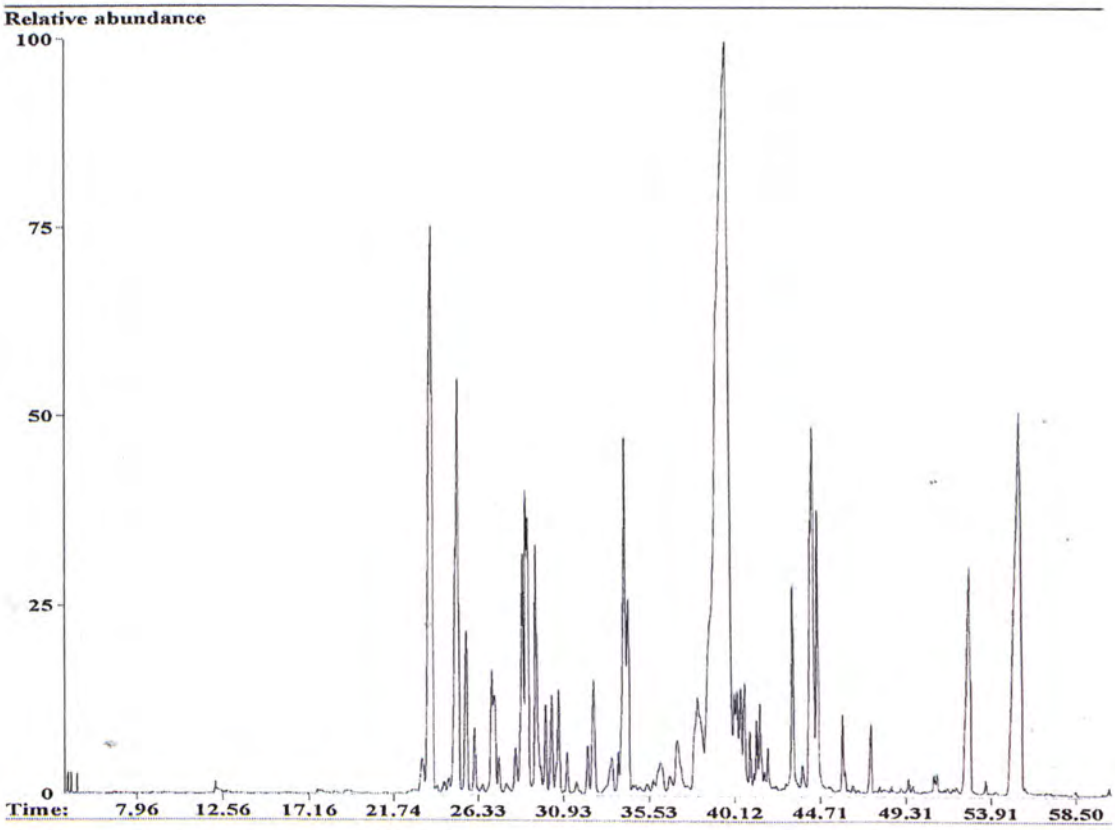

Figure A-1. Gas chromatogram of sample AL1

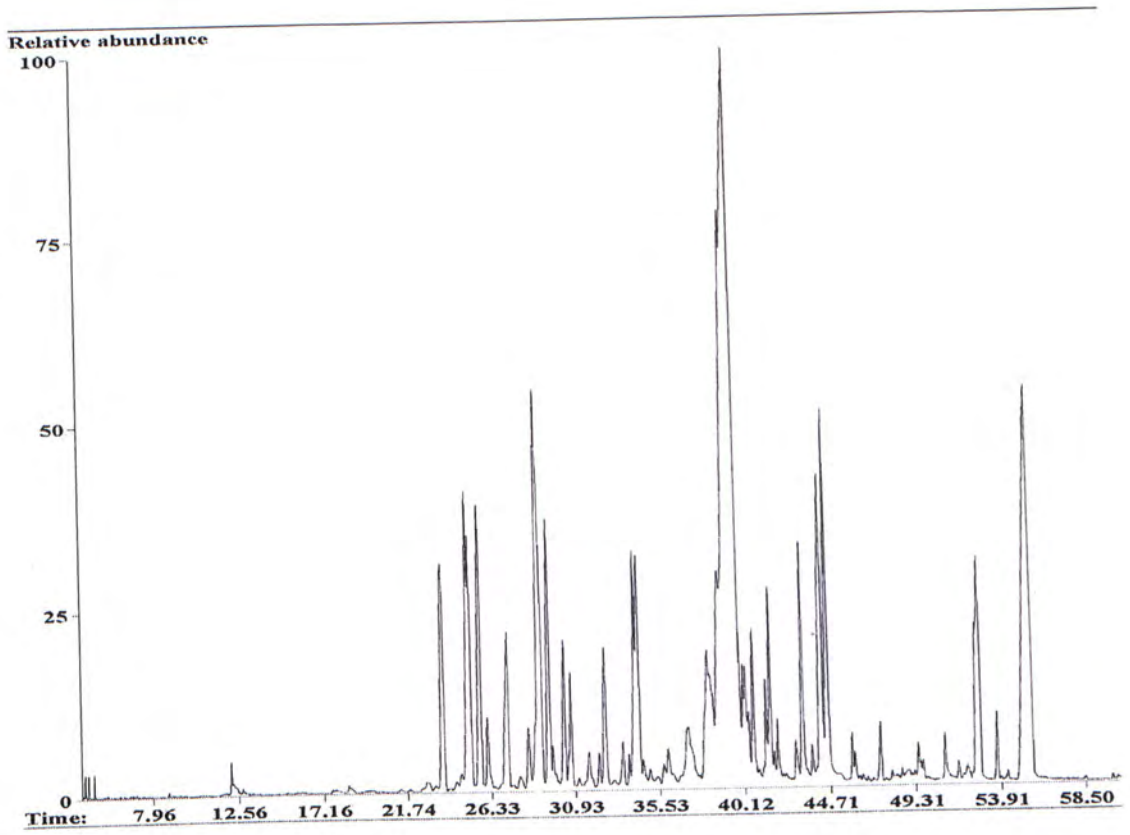

Figure A-2. Gas chromatogram of sample AL11

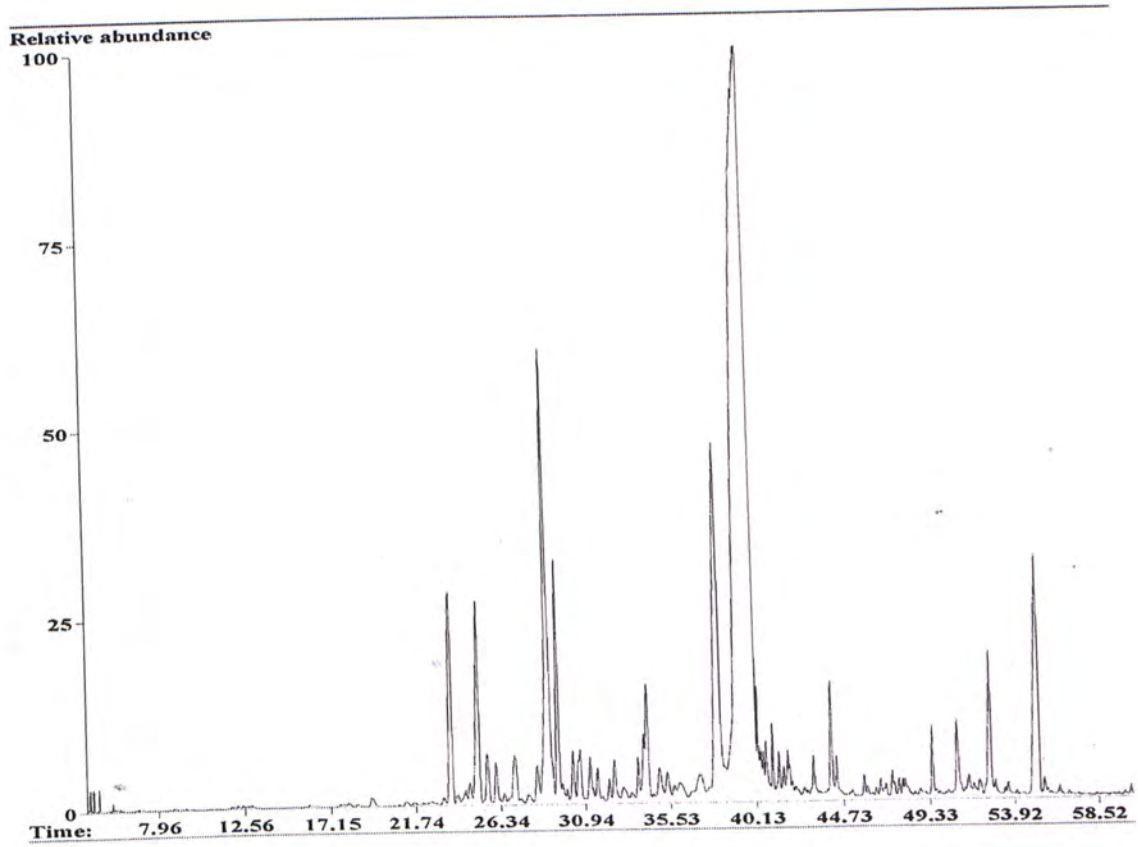

Figure A-3. Gas chromatogram of sample AL12

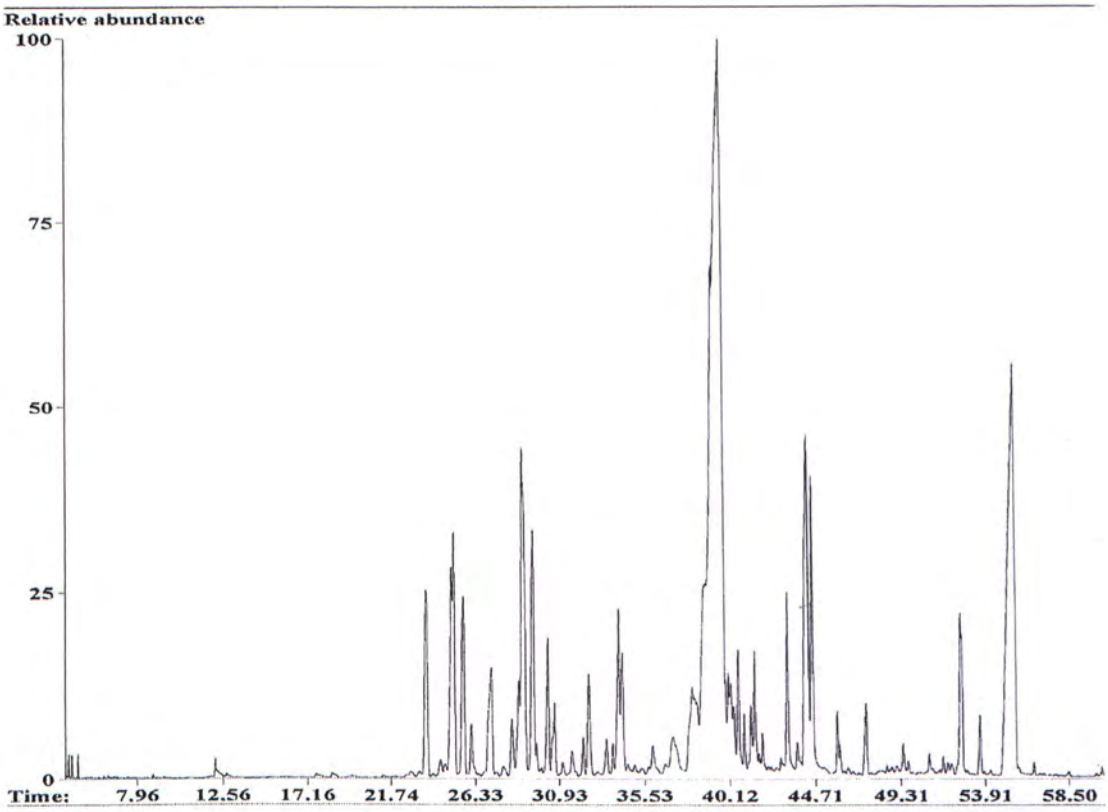

Figure A-4. Gas chromatogram of sample AL15

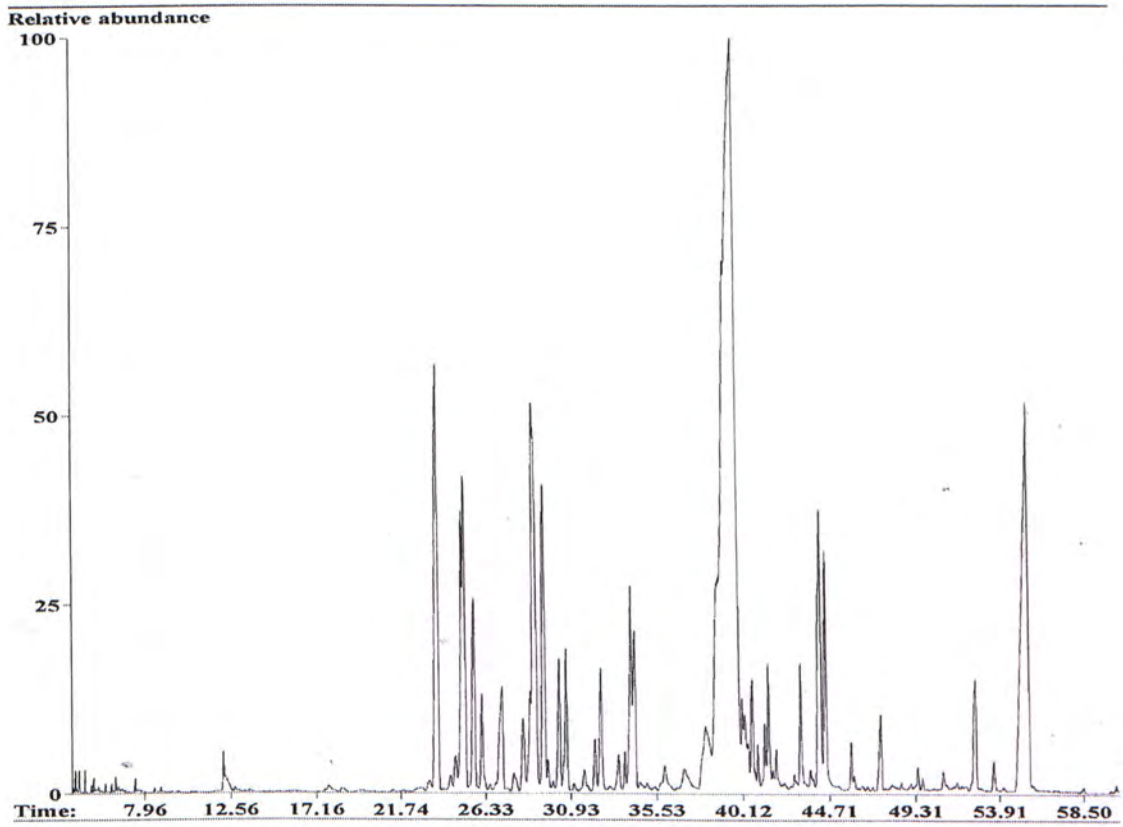

Figure A-5. Gas chromatogram of sample AL16

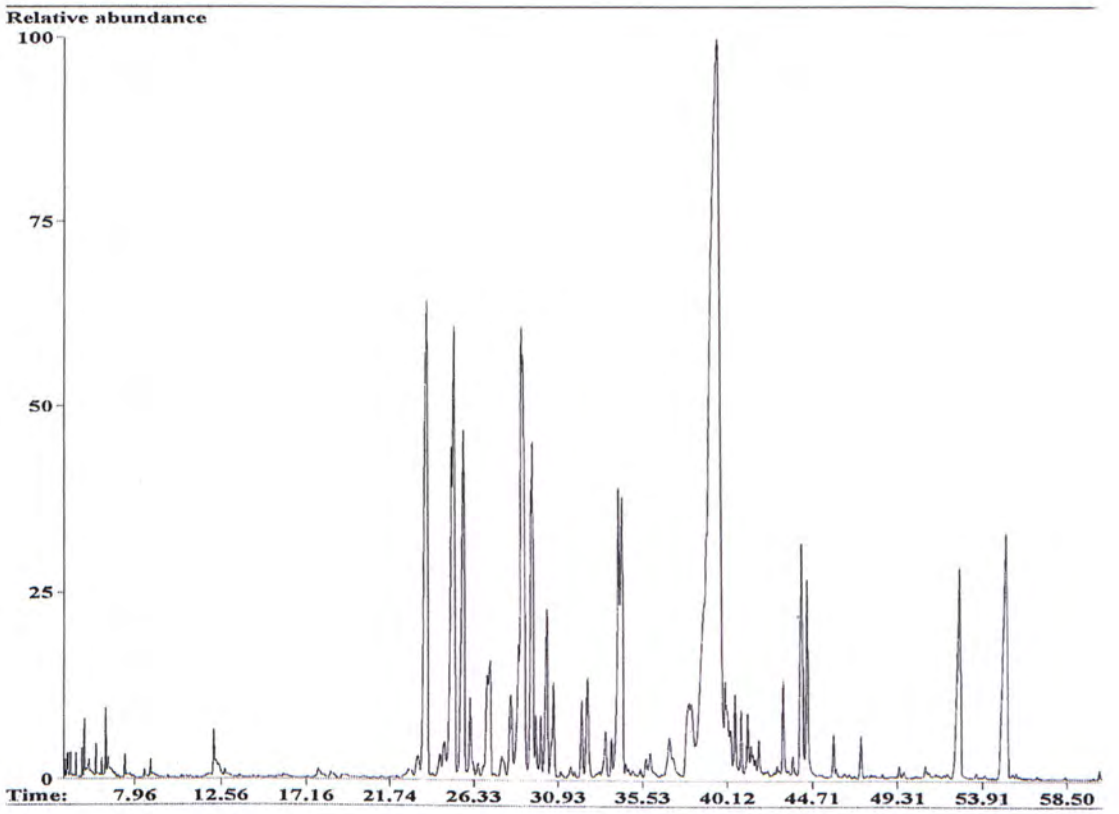

Figure A-6. Gas chromatogram of sample AL21

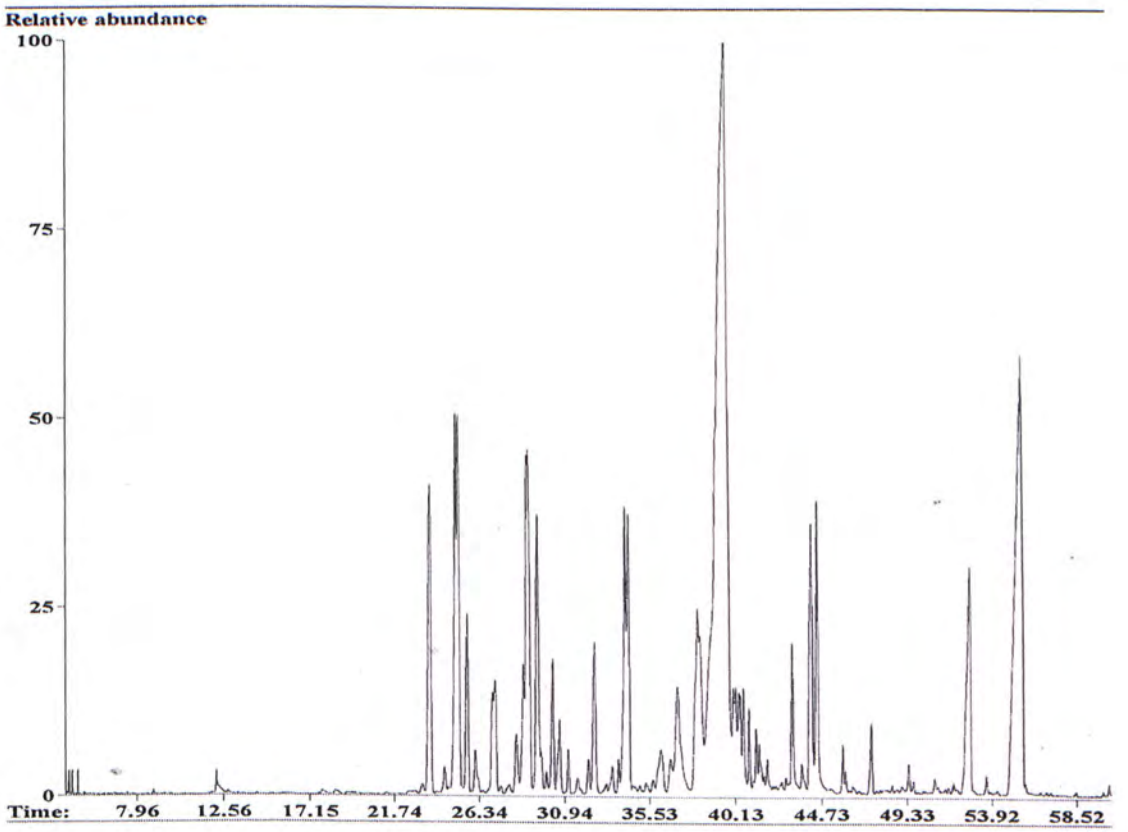

Figure A-7. Gas chromatogram of sample AL22

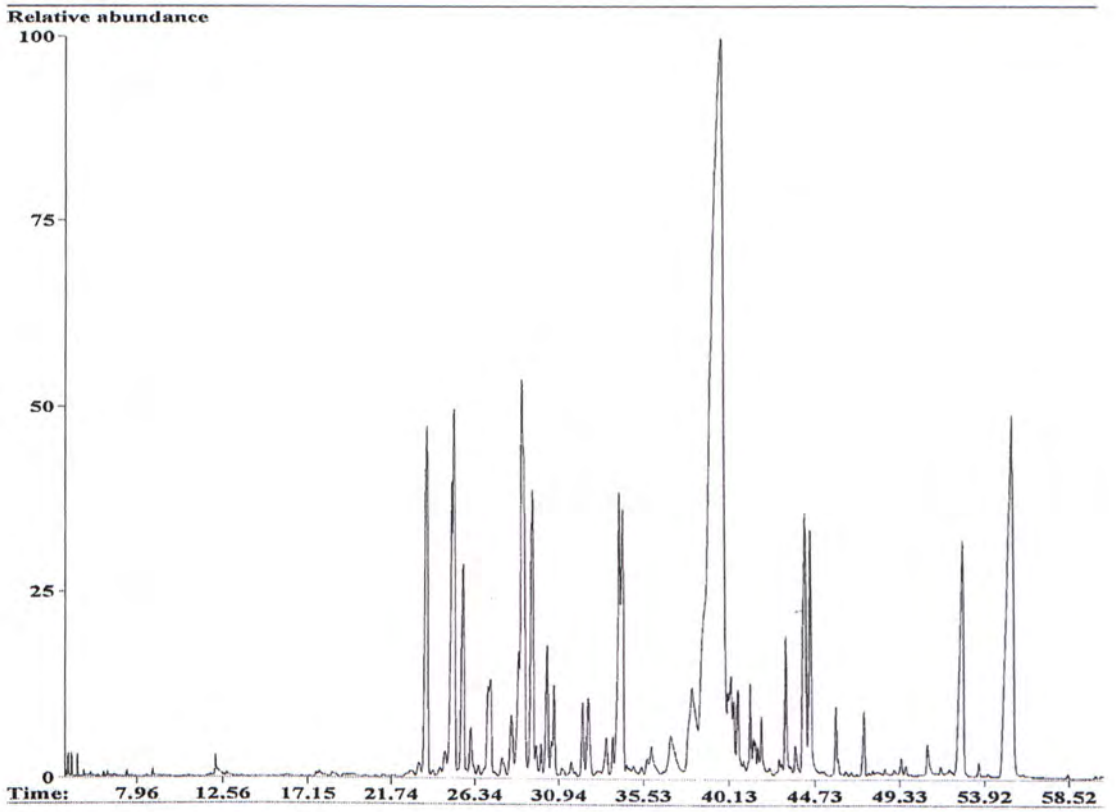

Figure A-8. Gas chromatogram of sample AL23

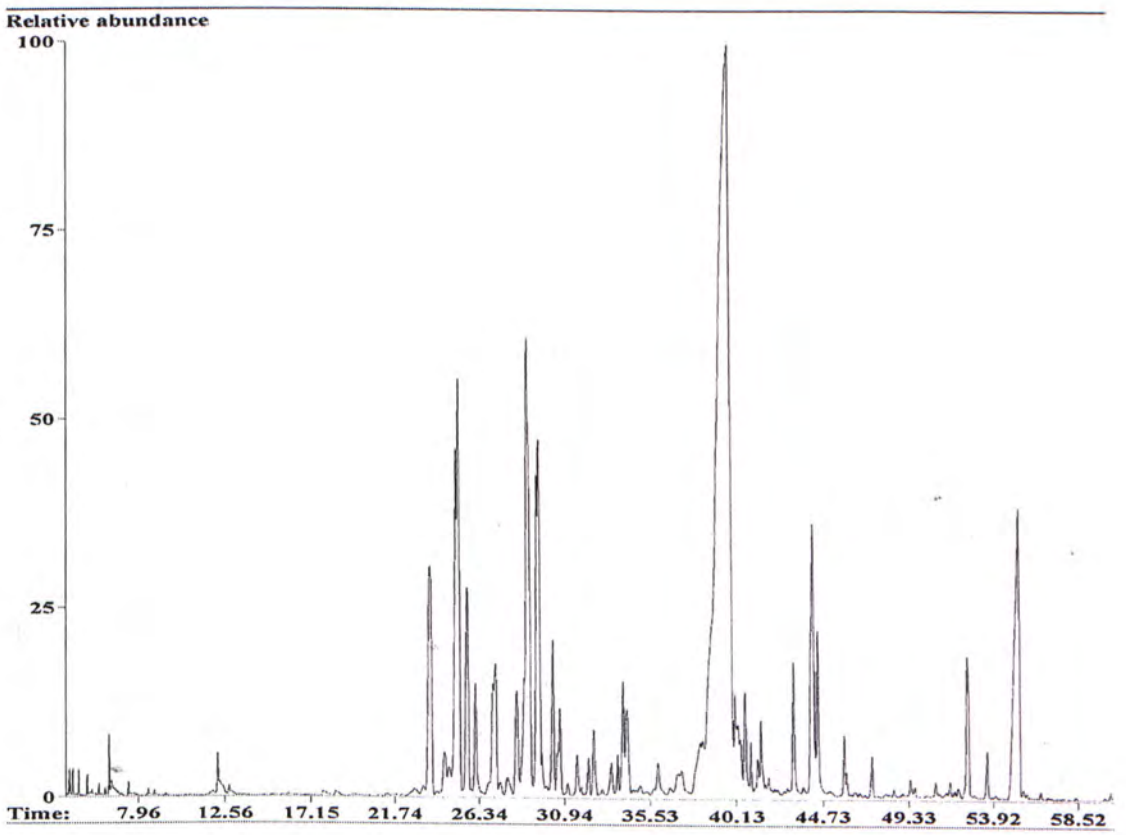

Figure A-9. Gas chromatogram of sample AL25

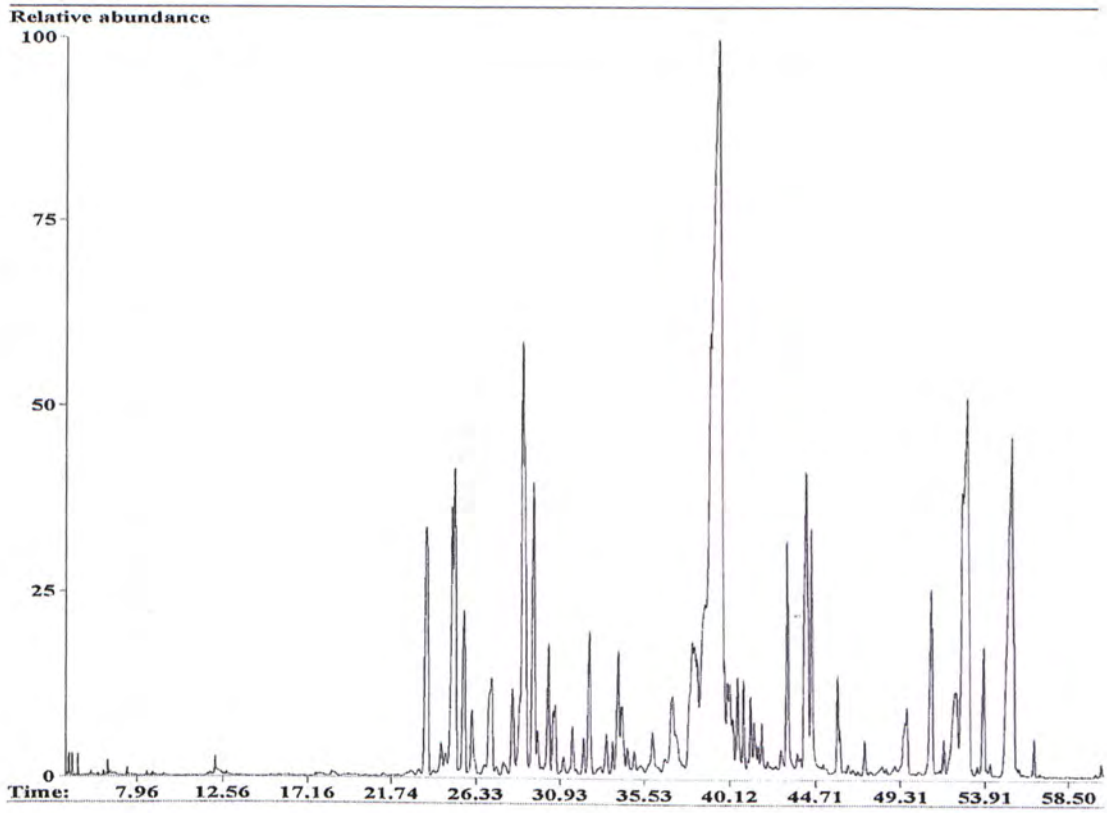

Figure A-10. Gas chromatogram of sample AL25a

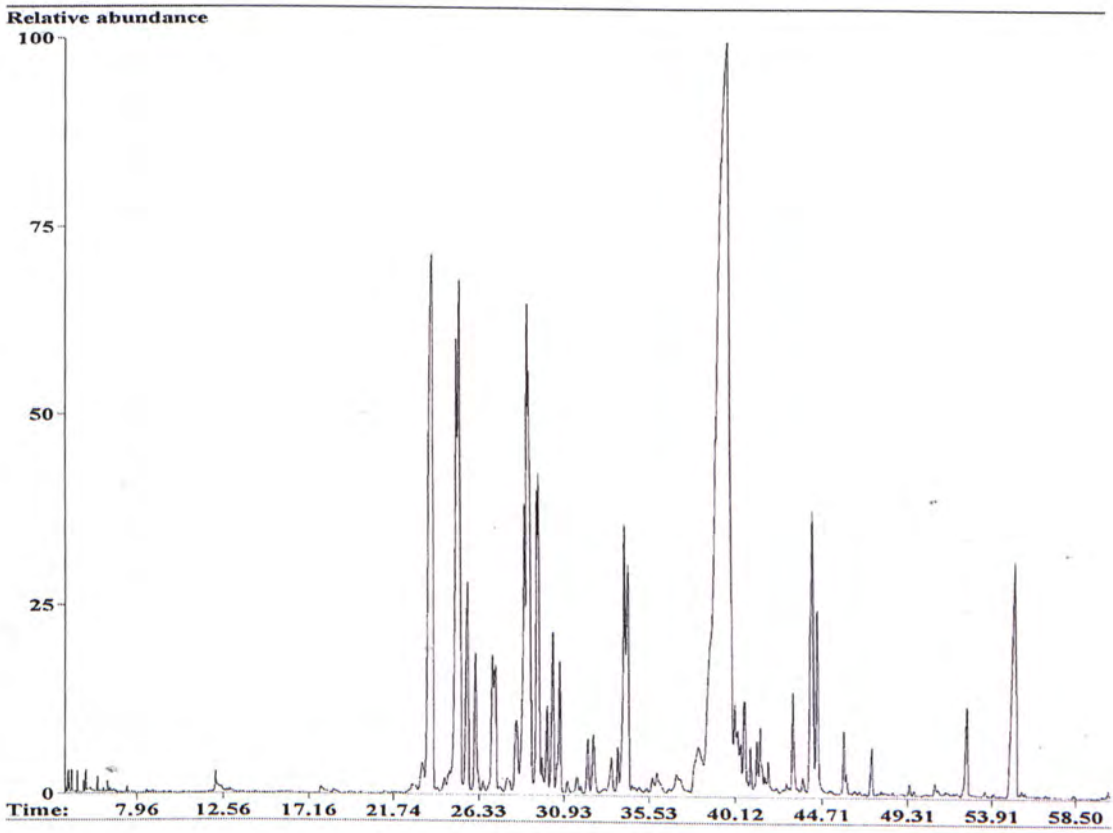

Figure A-11. Gas chromatogram of sample AL27

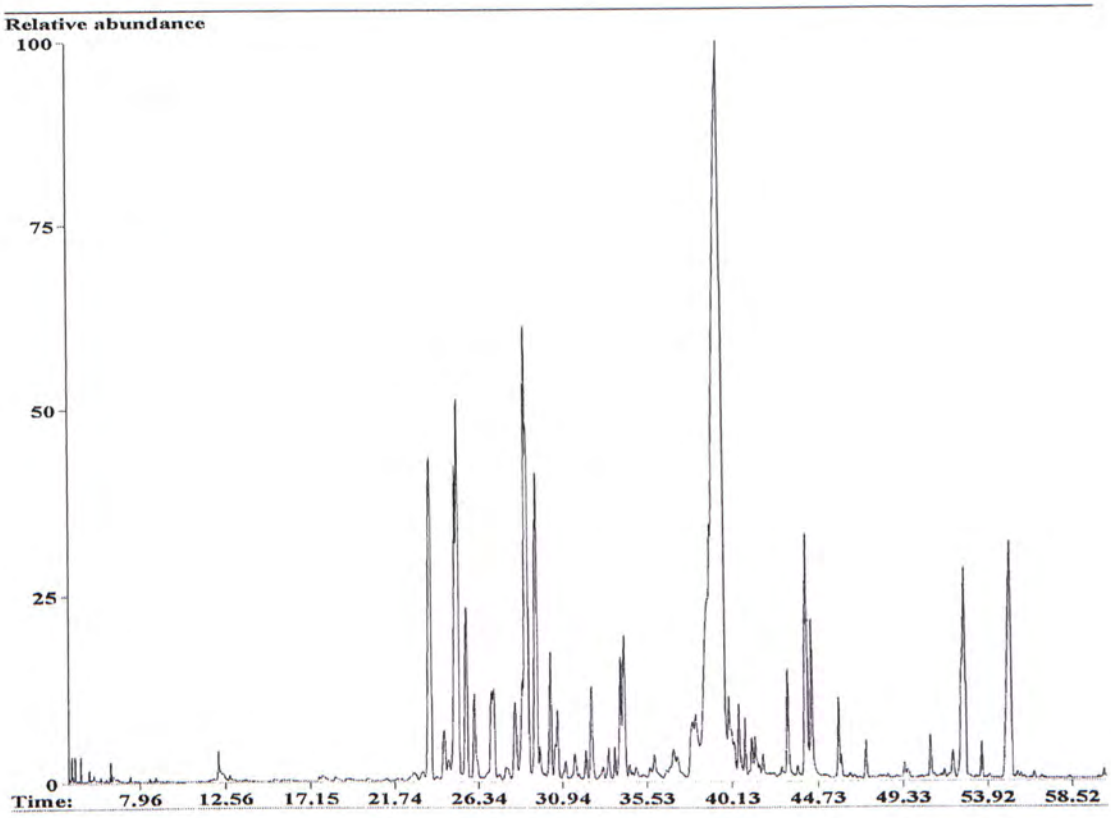

Figure A-12. Gas chromatogram of sample AL28

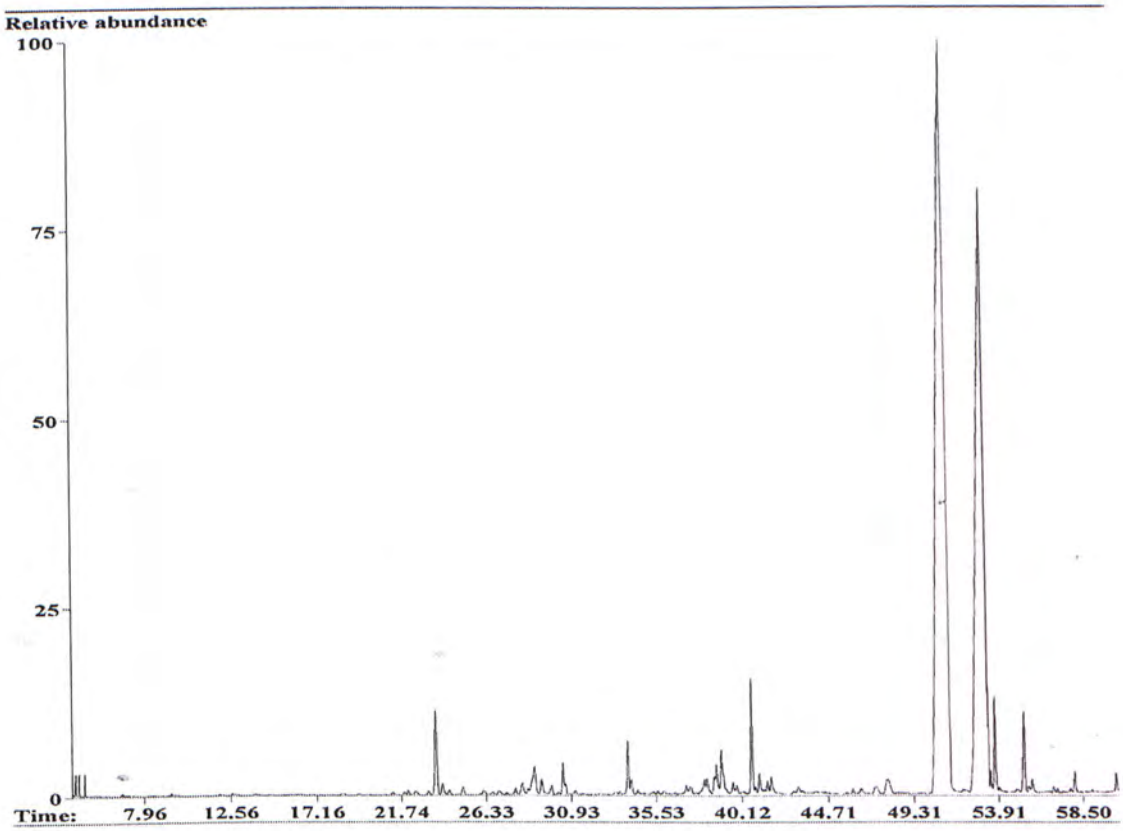

Figure A-13. Gas chromatogram of sample IH02

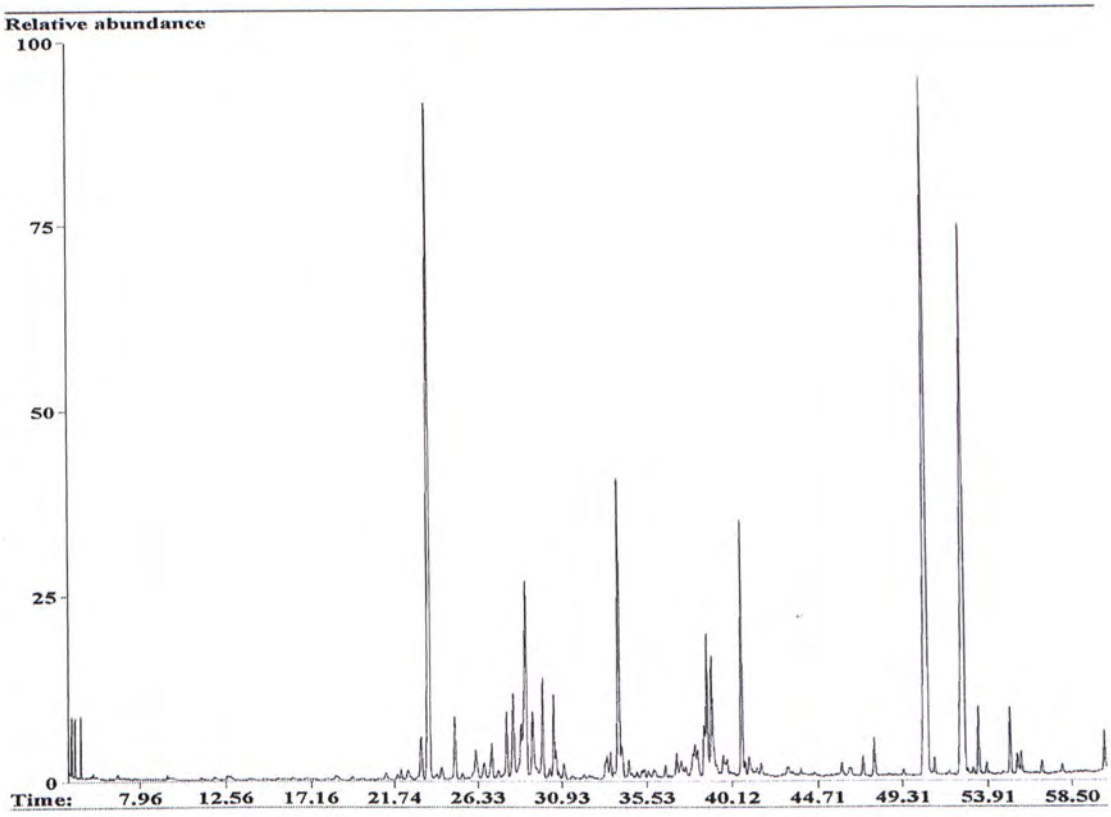

Figure A-14. Gas chromatogram of sample IH07

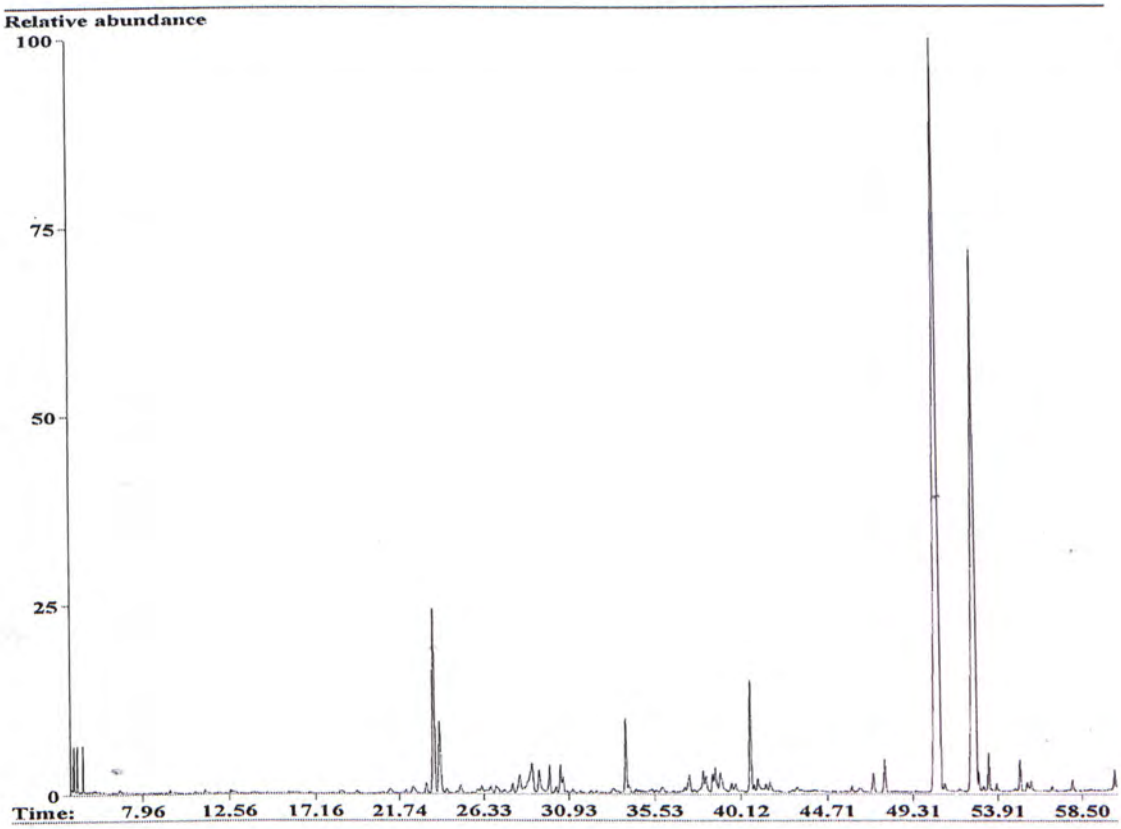

Figure A-15. Gas chromatogram of sample IH08

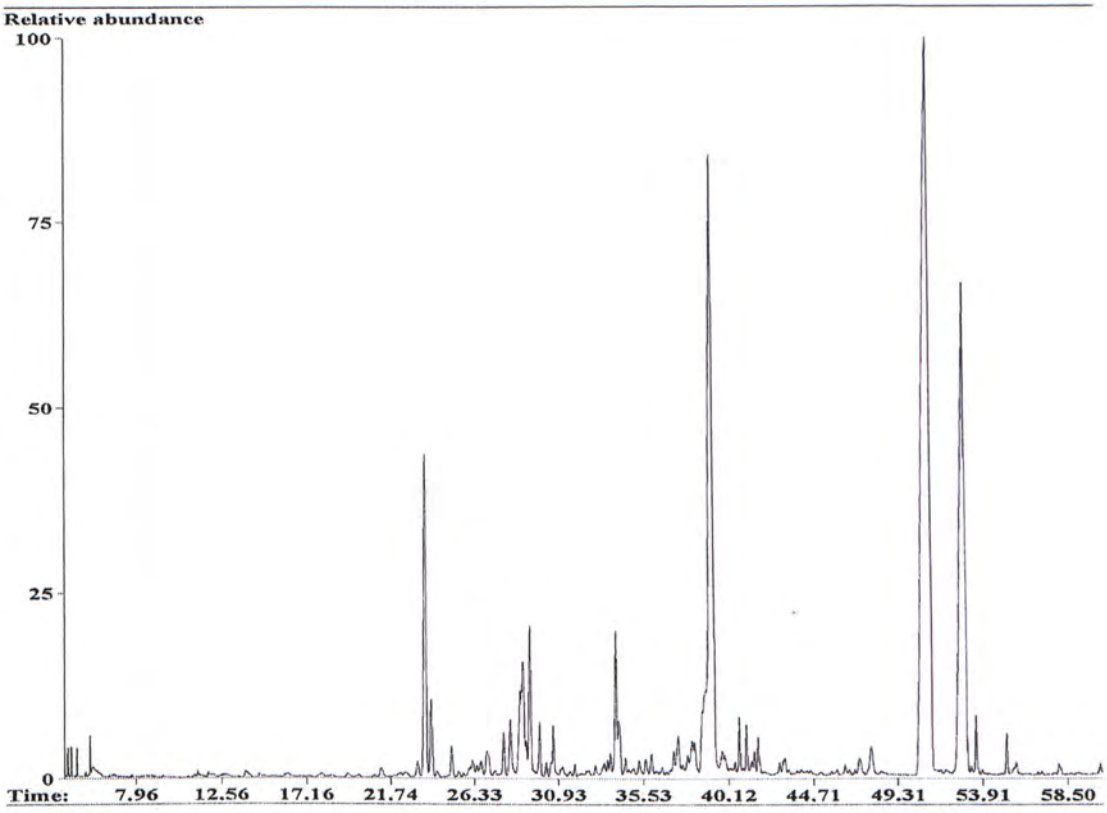

Figure A-16. Gas chromatogram of sample IH11

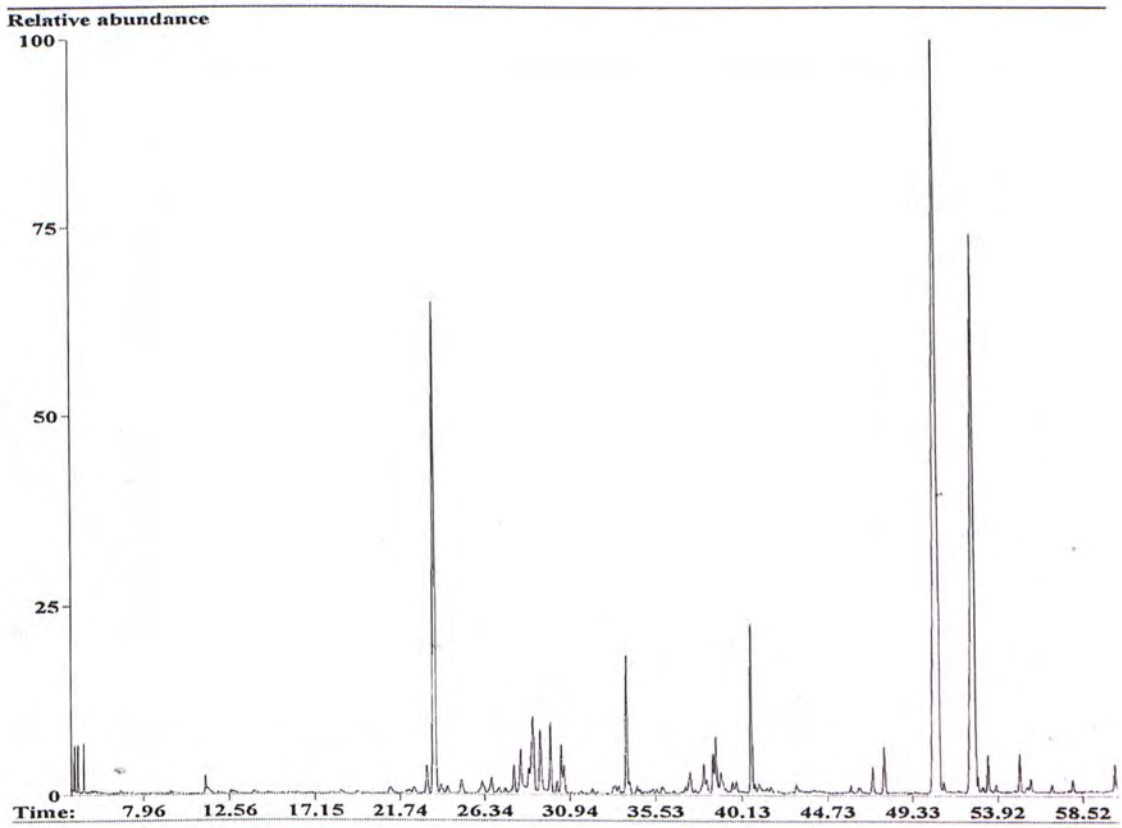

Figure A-17. Gas chromatogram of sample IH12

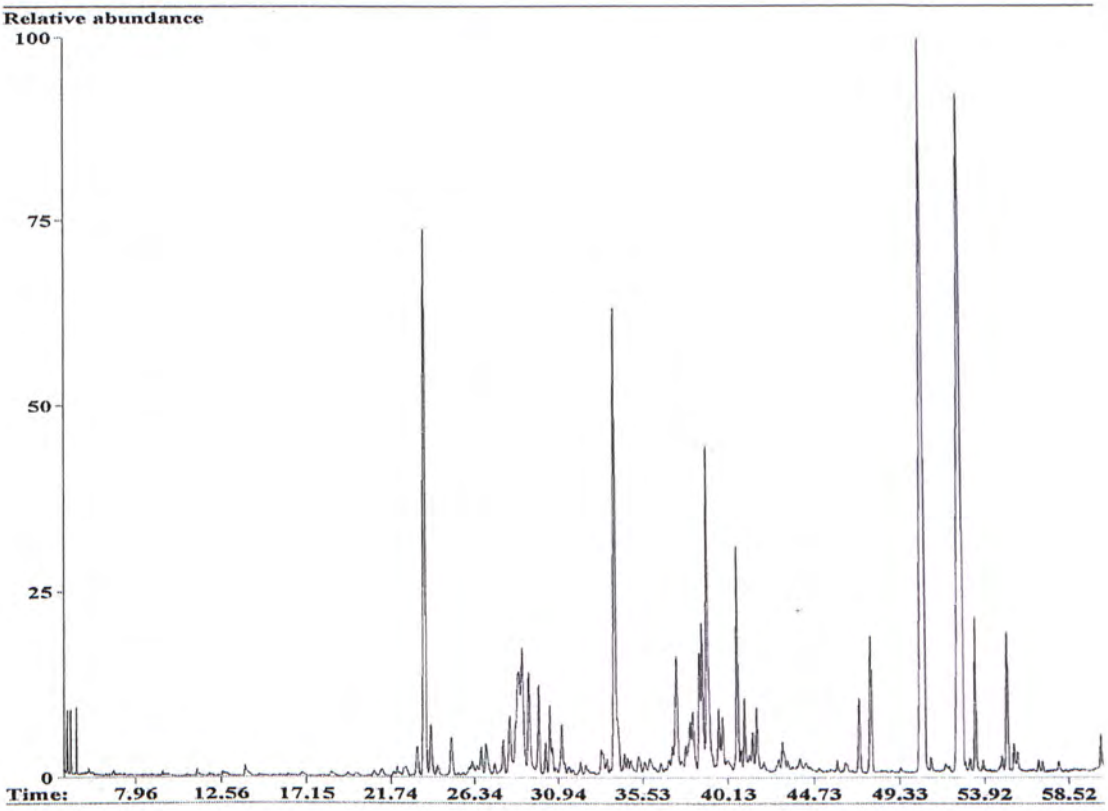

Figure A-18. Gas chromatogram of sample IH13

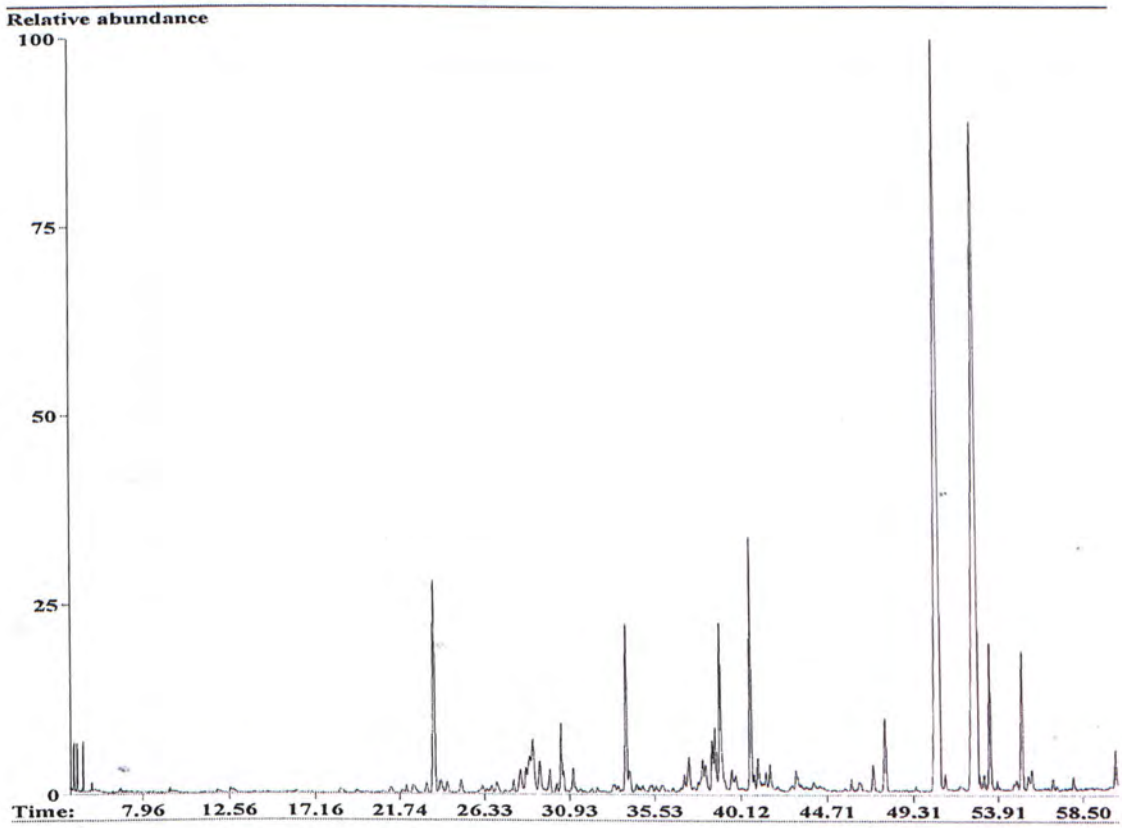

Figure A-19. Gas chromatogram of sample IH16

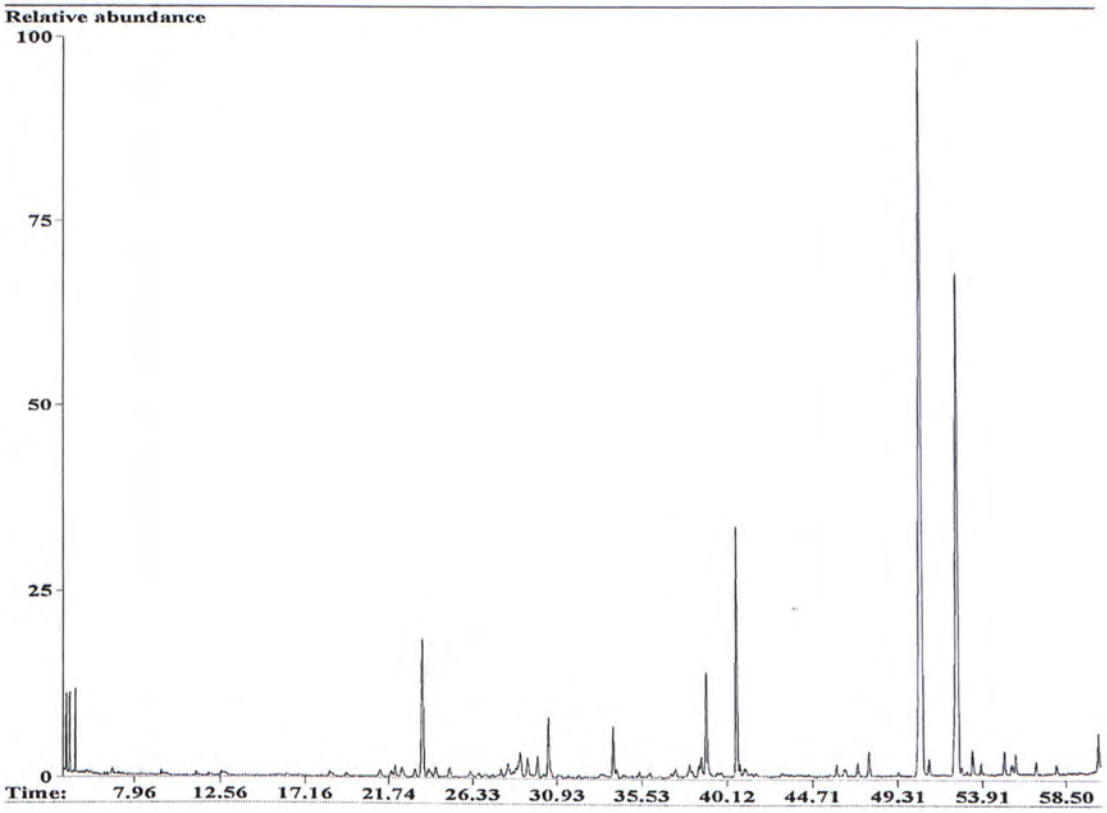

Figure A-20. Gas chromatogram of sample IH17

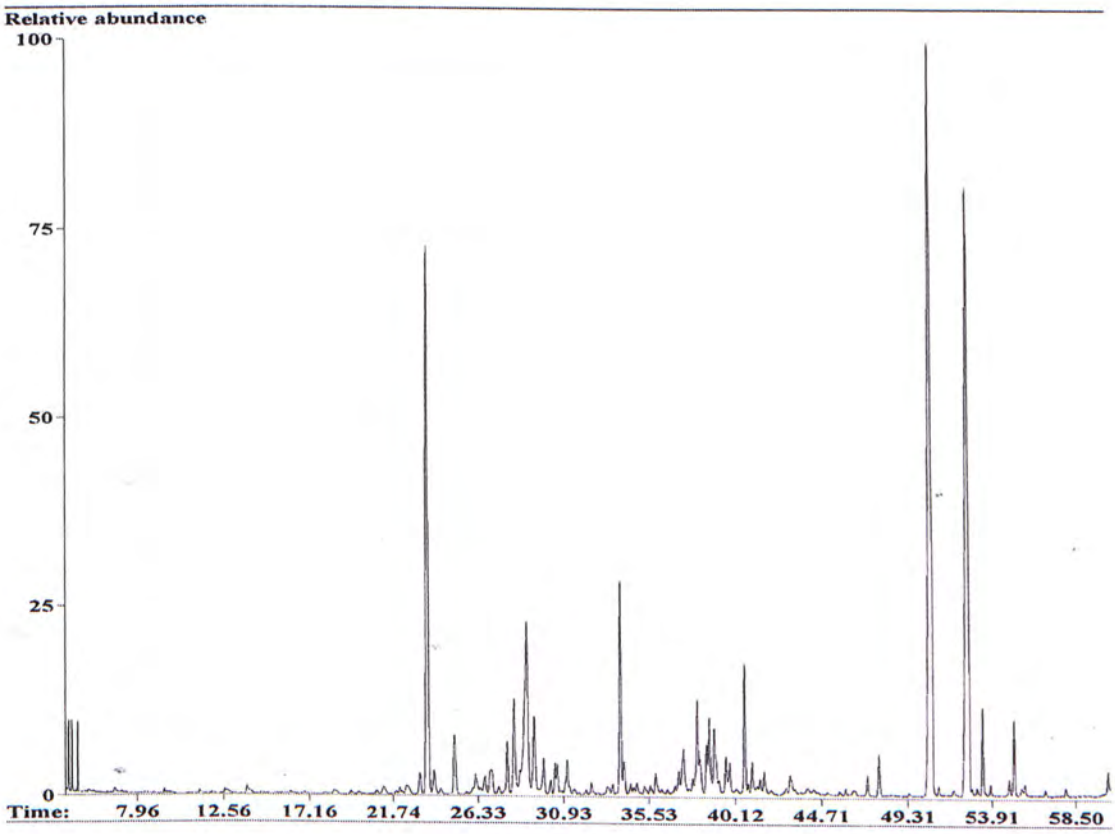

Figure A-21. Gas chromatogram of sample IH18

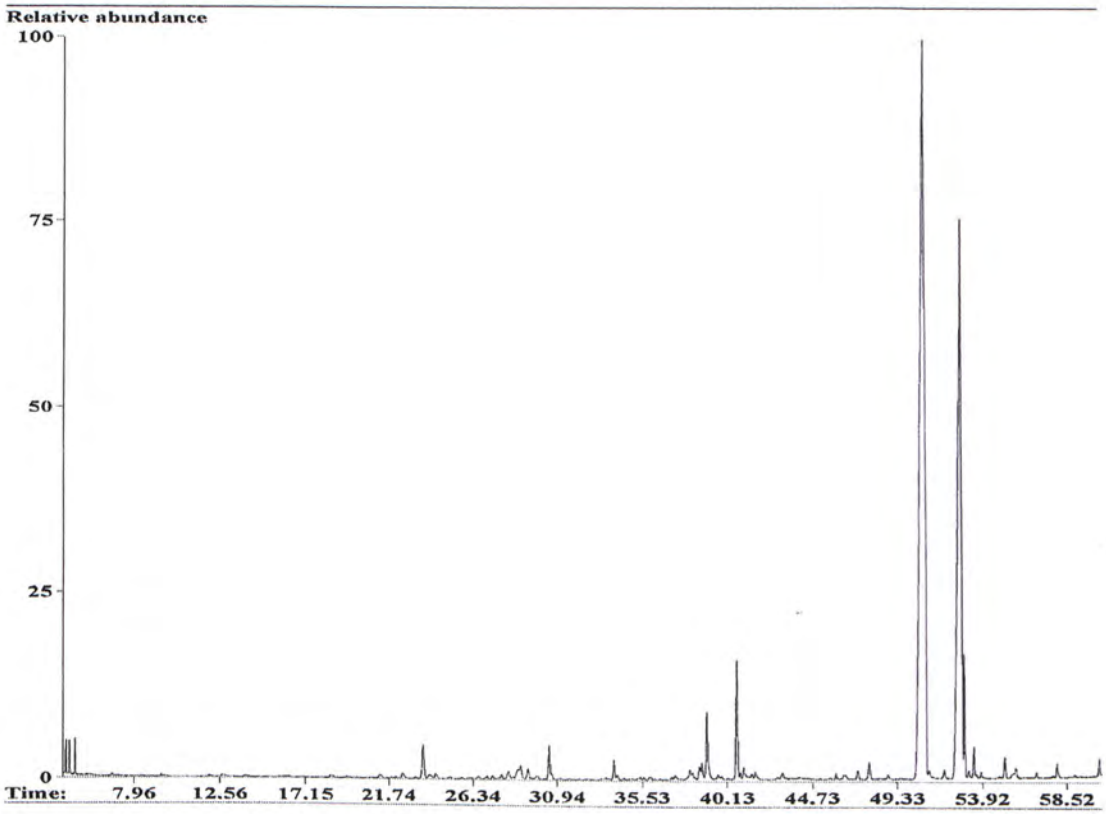

Figure A-22. Gas chromatogram of sample IR01

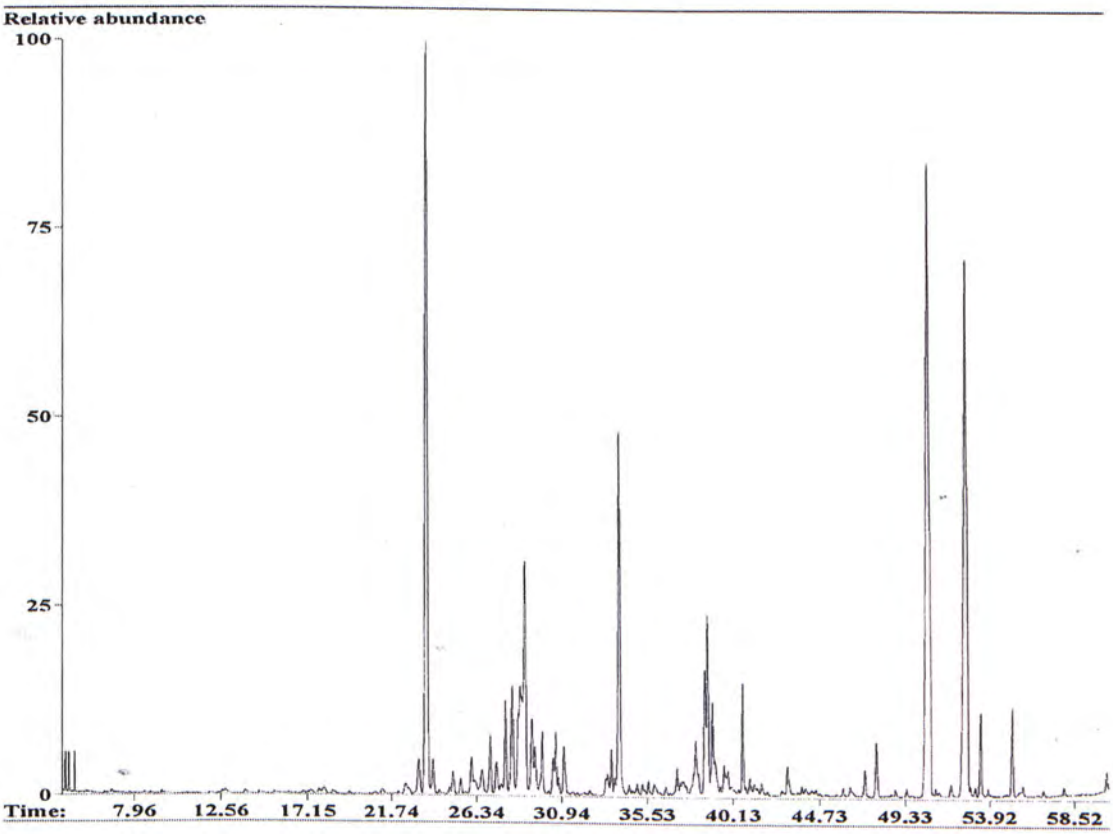

Figure A-23. Gas chromatogram of sample IR03

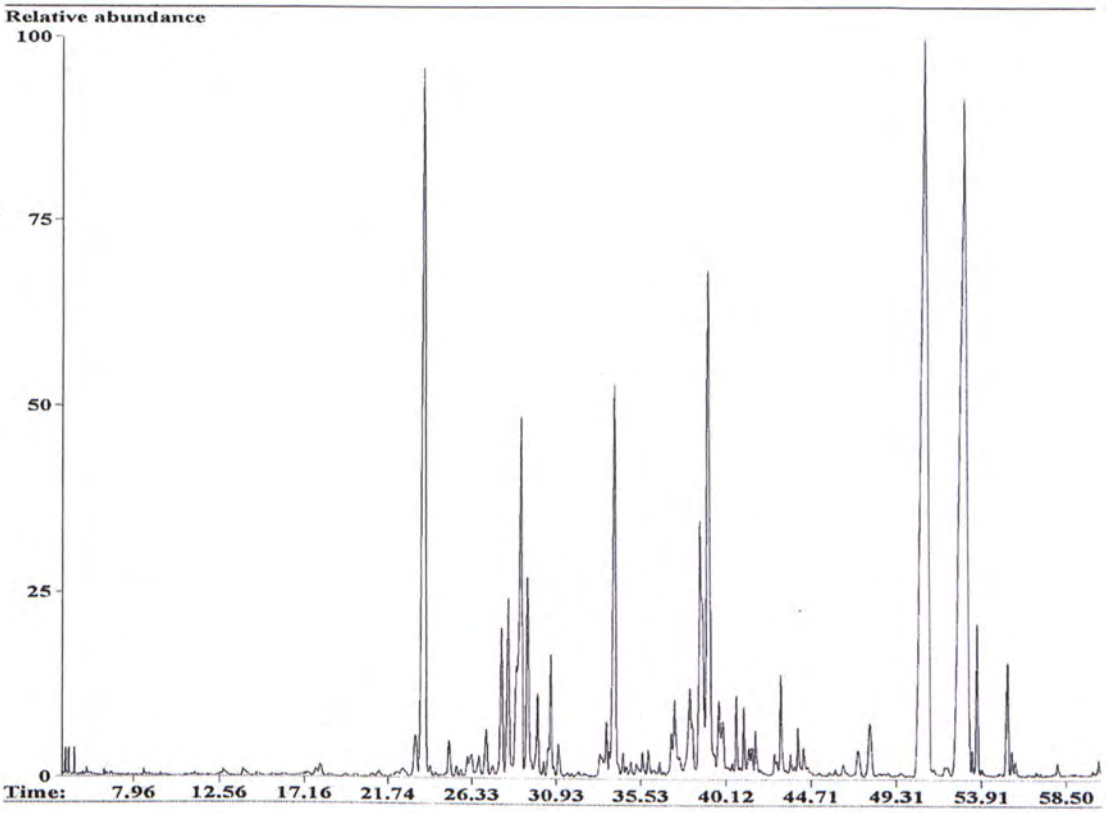

Figure A-24. Gas chromatogram of sample IR05

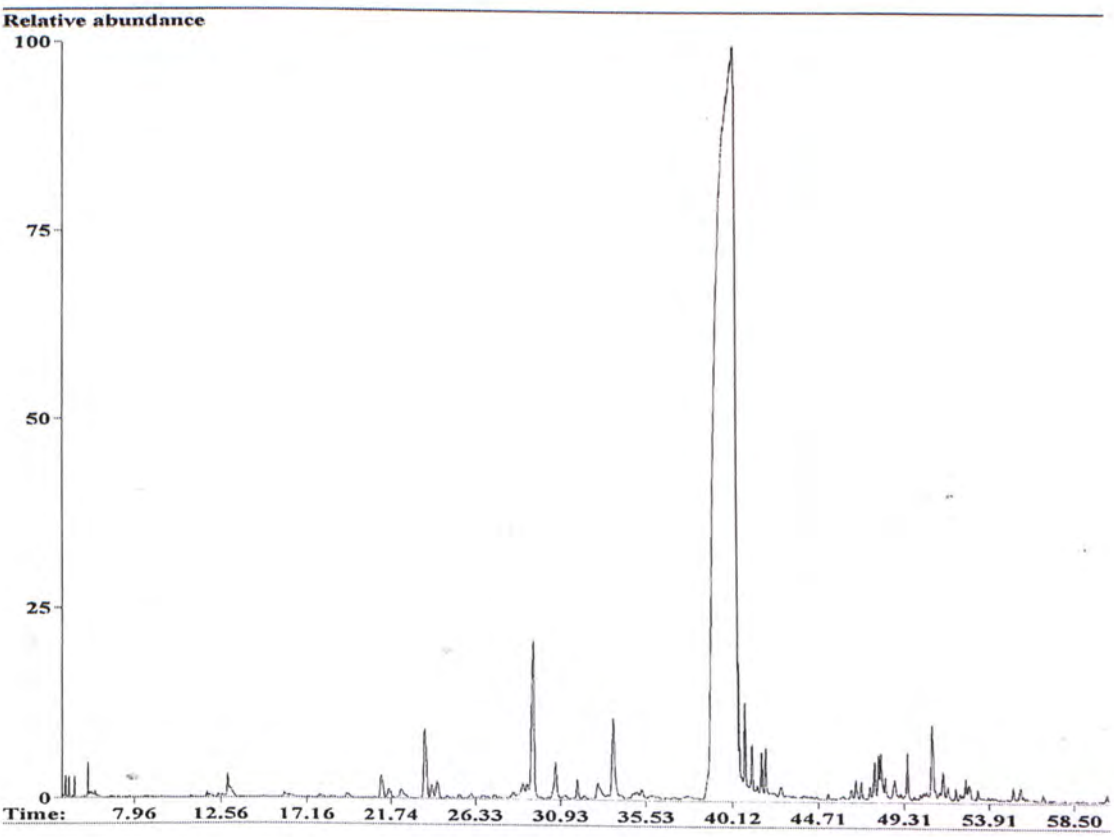

Figure A-25. Gas chromatogram of sample VB01

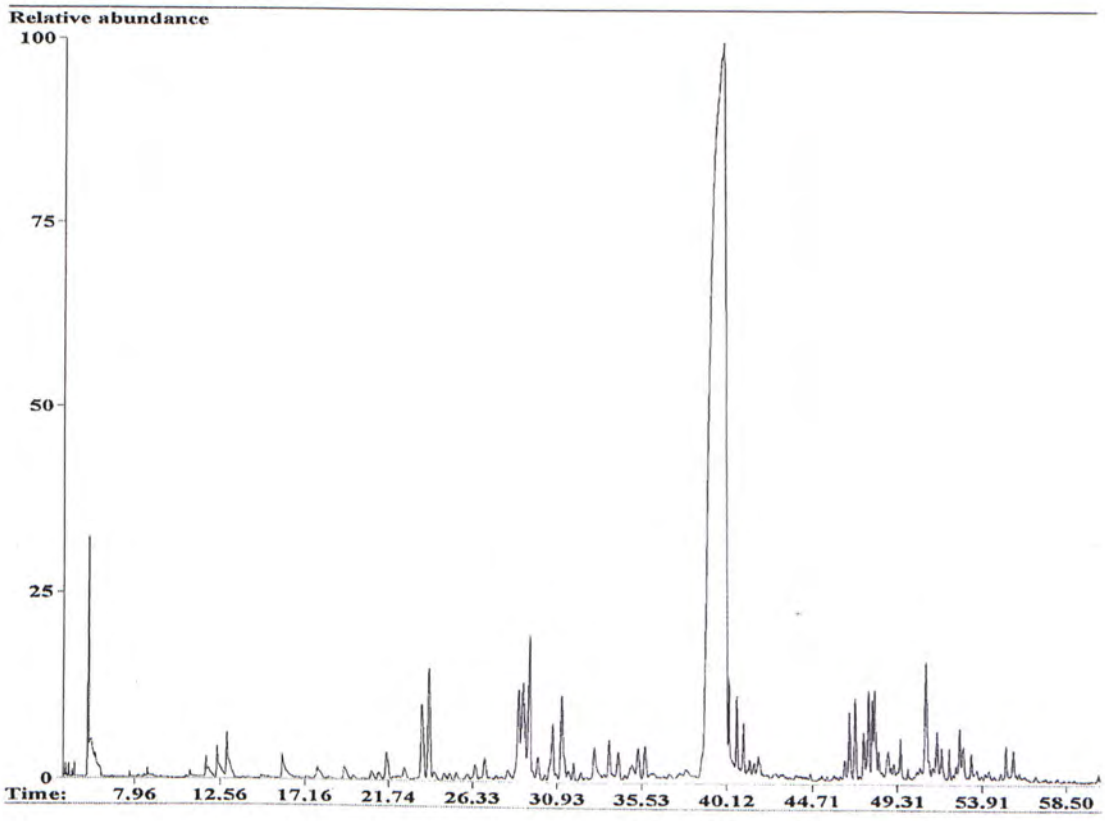

Figure A-26. Gas chromatogram of sample VB02

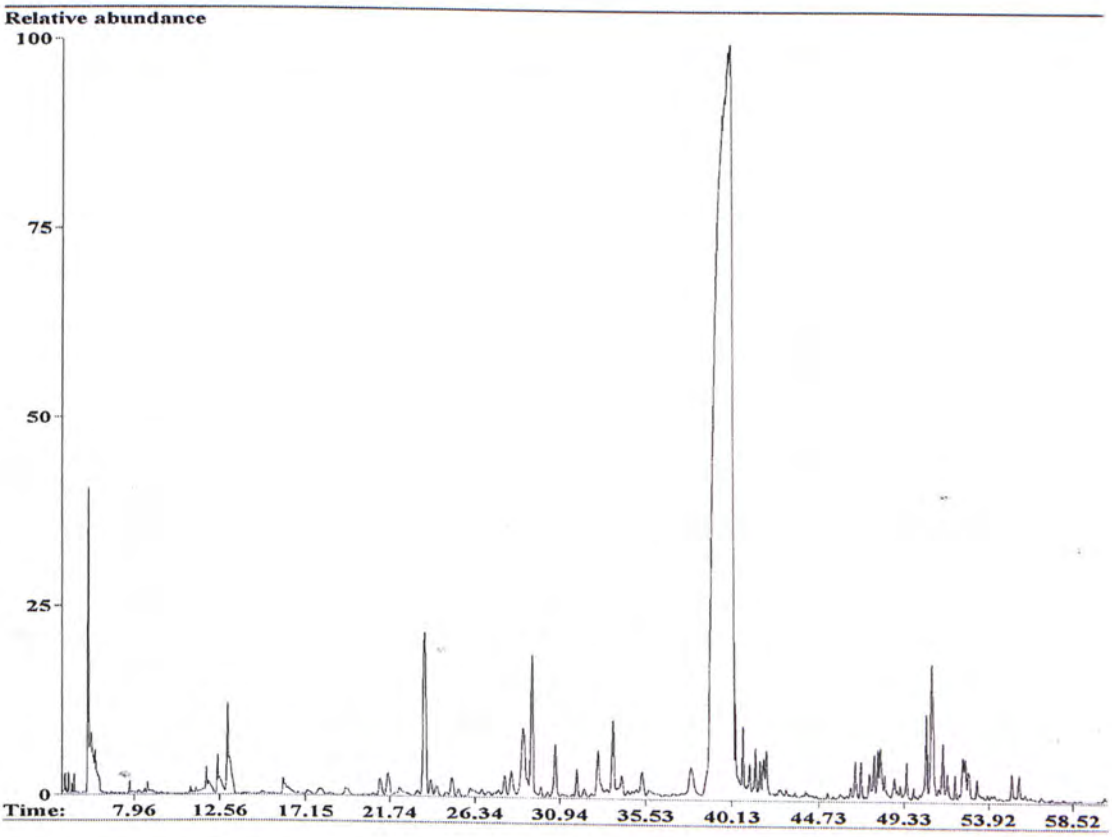

Figure A-27. Gas chromatogram of sample VB05

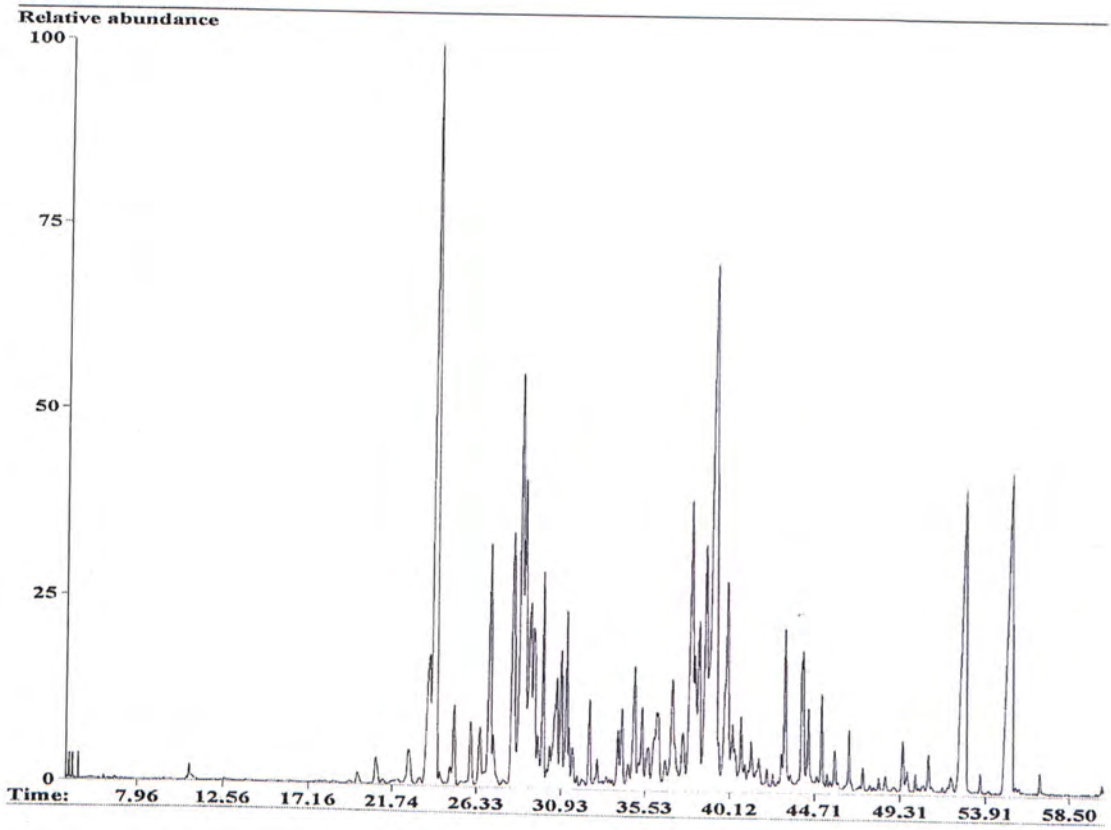

Figure A-28. Gas chromatogram of sample VS02

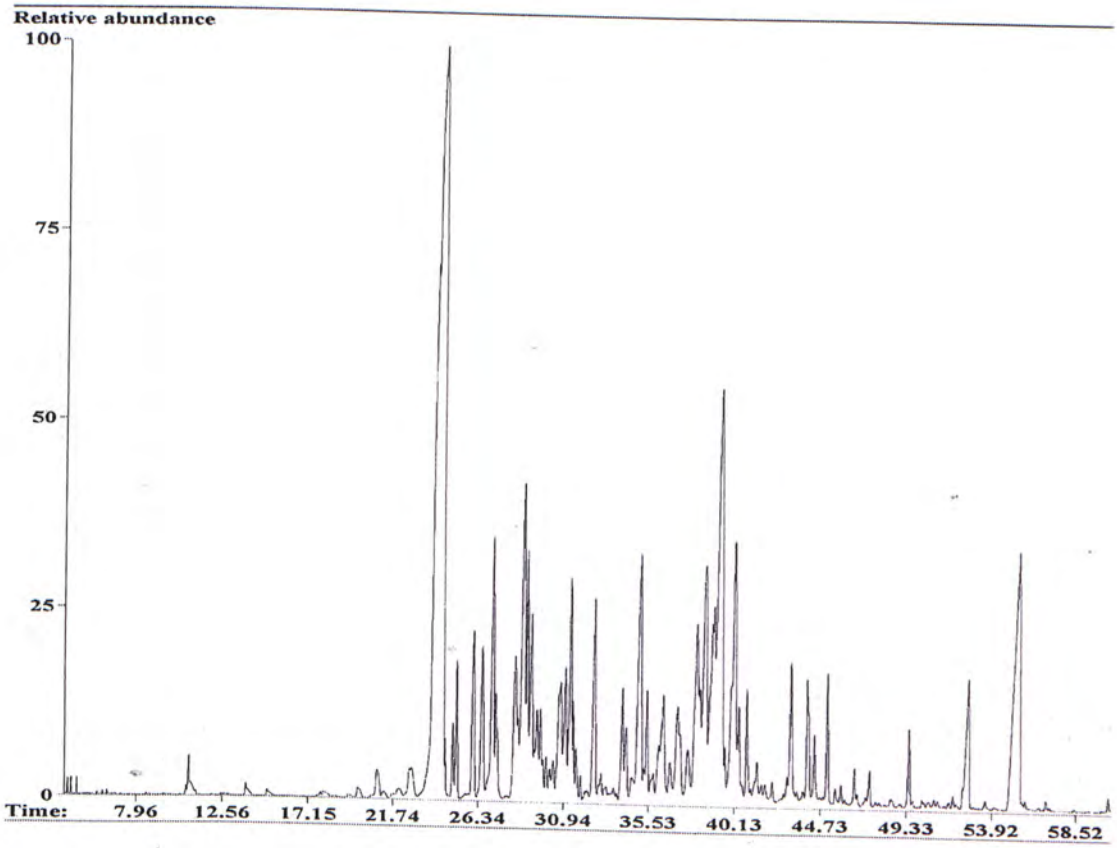

Figure A-29. Gas chromatogram of sample VS07

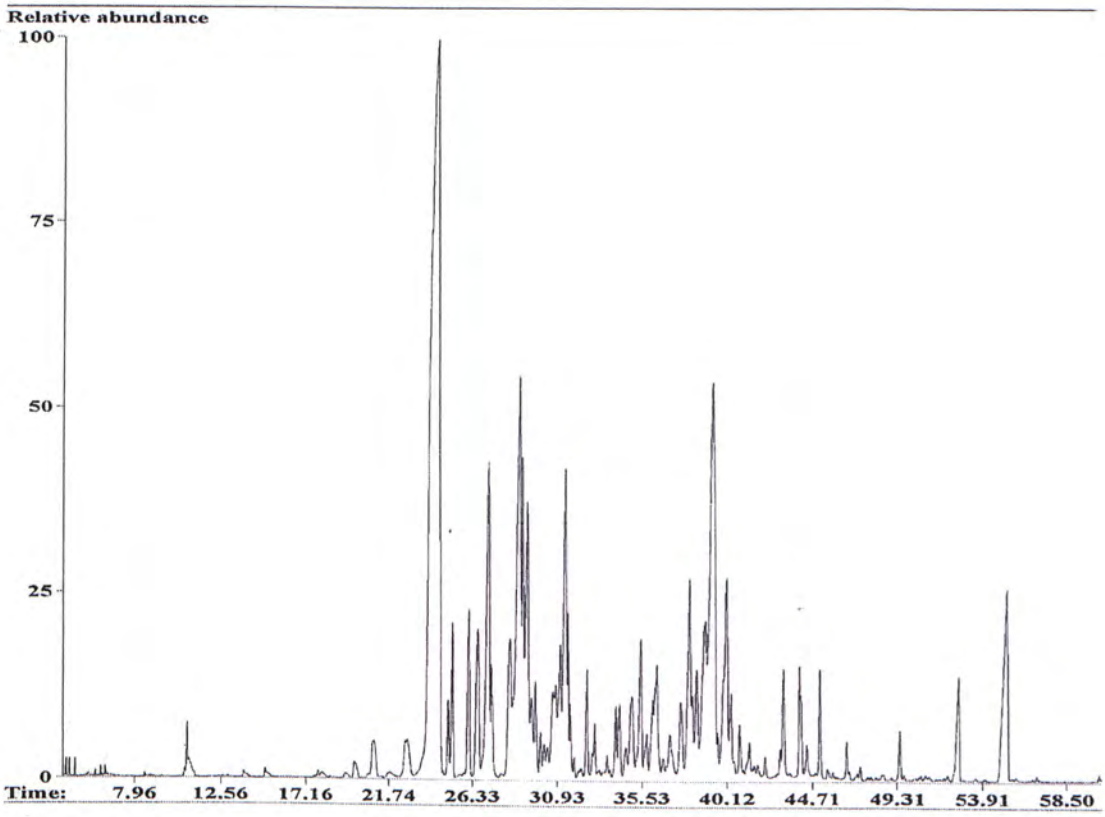

Figure A-30. Gas chromatogram of sample VS08

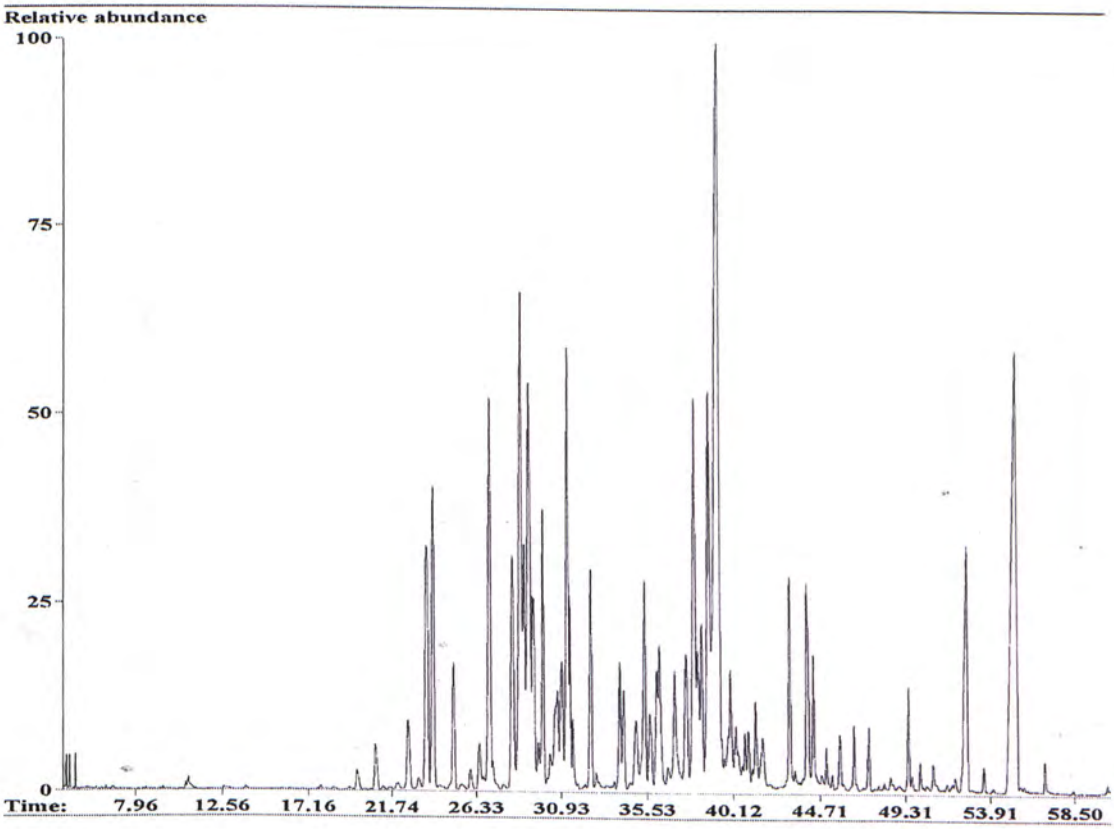

Figure A-31. Gas chromatogram of sample VS09

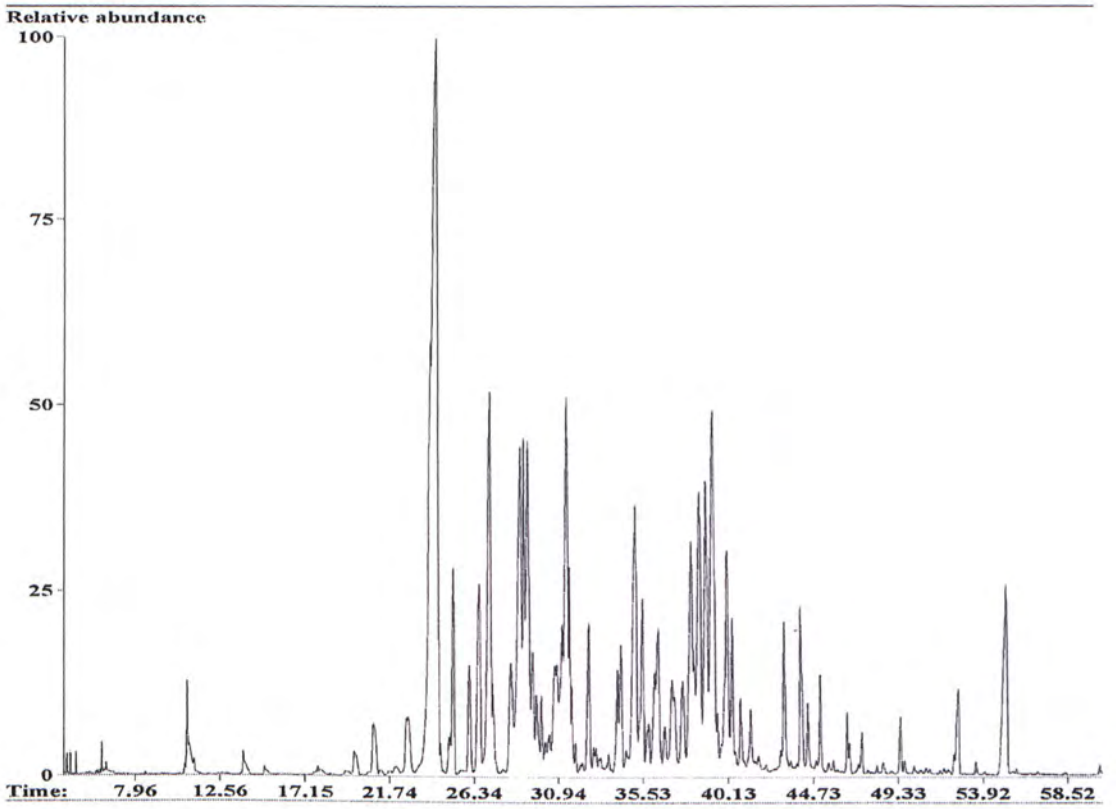

Figure A-32. Gas chromatogram of sample VS10

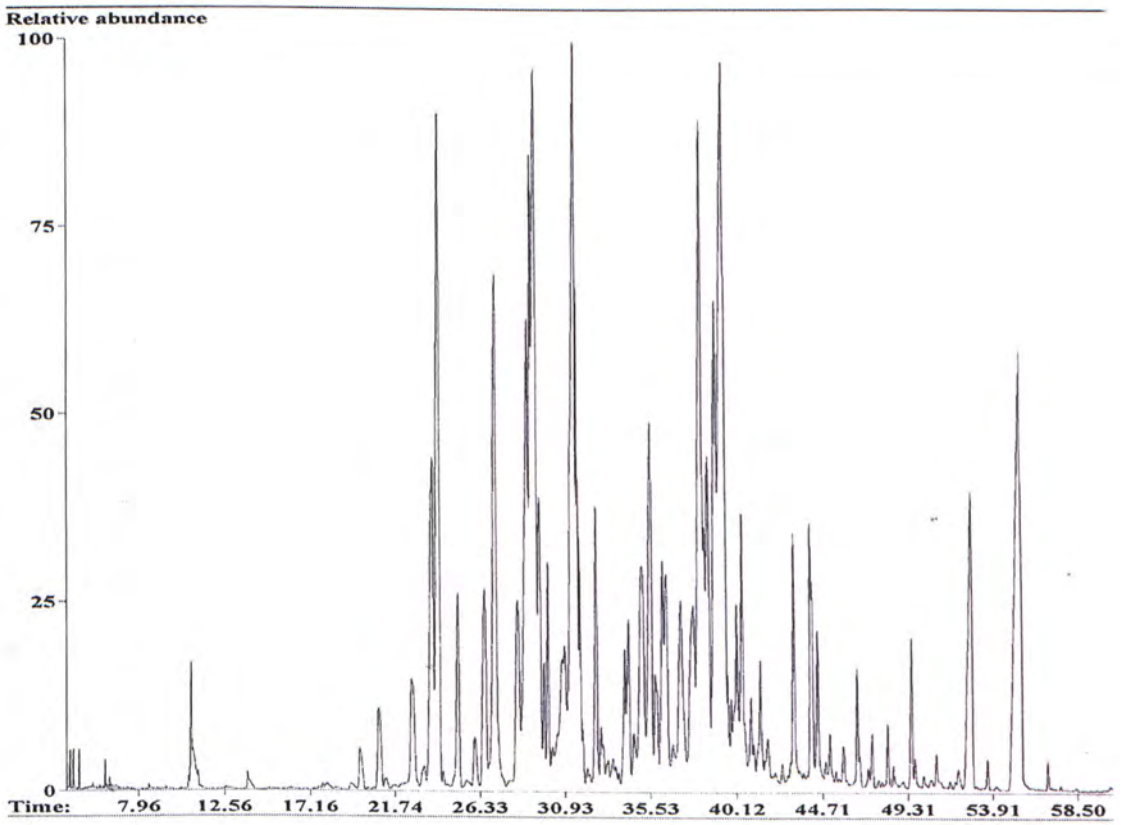

Figure A-33. Gas chromatogram of sample VS12

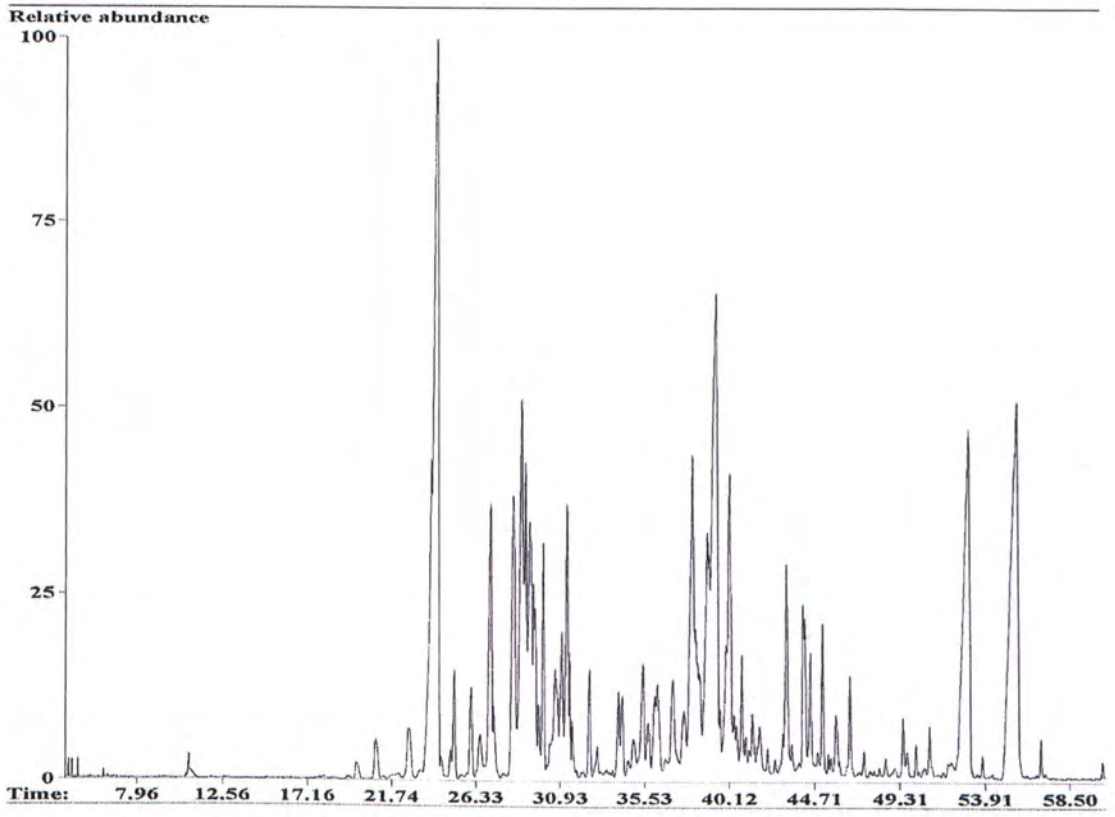

Figure A-34. Gas chromatogram of sample VS13

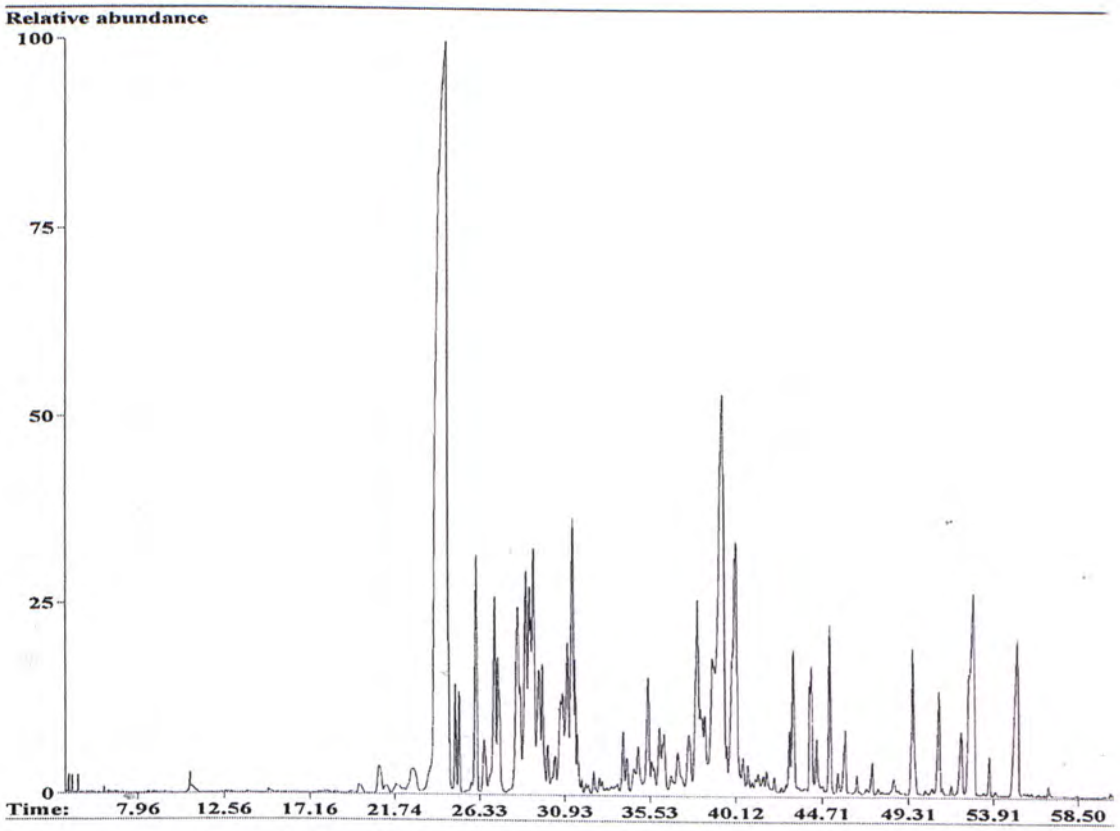

Figure A-35. Gas chromatogram of sample VS15

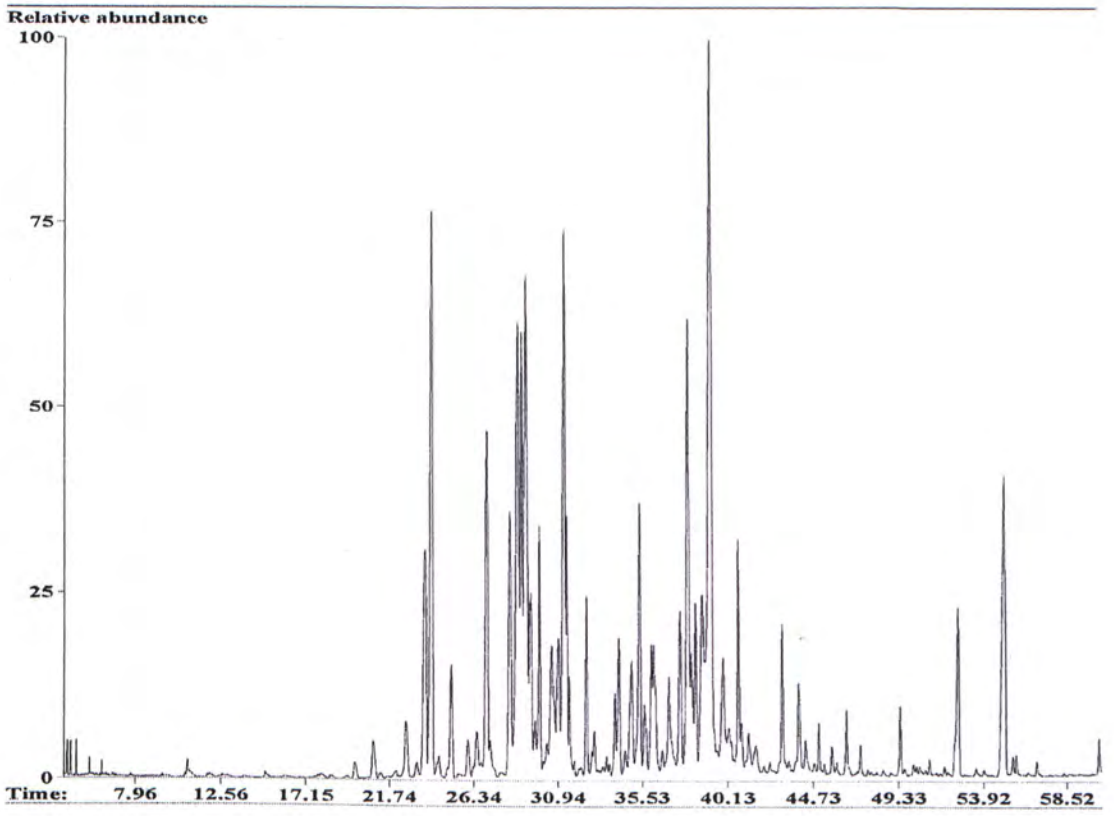

Figure A-36. Gas chromatogram of sample VS17

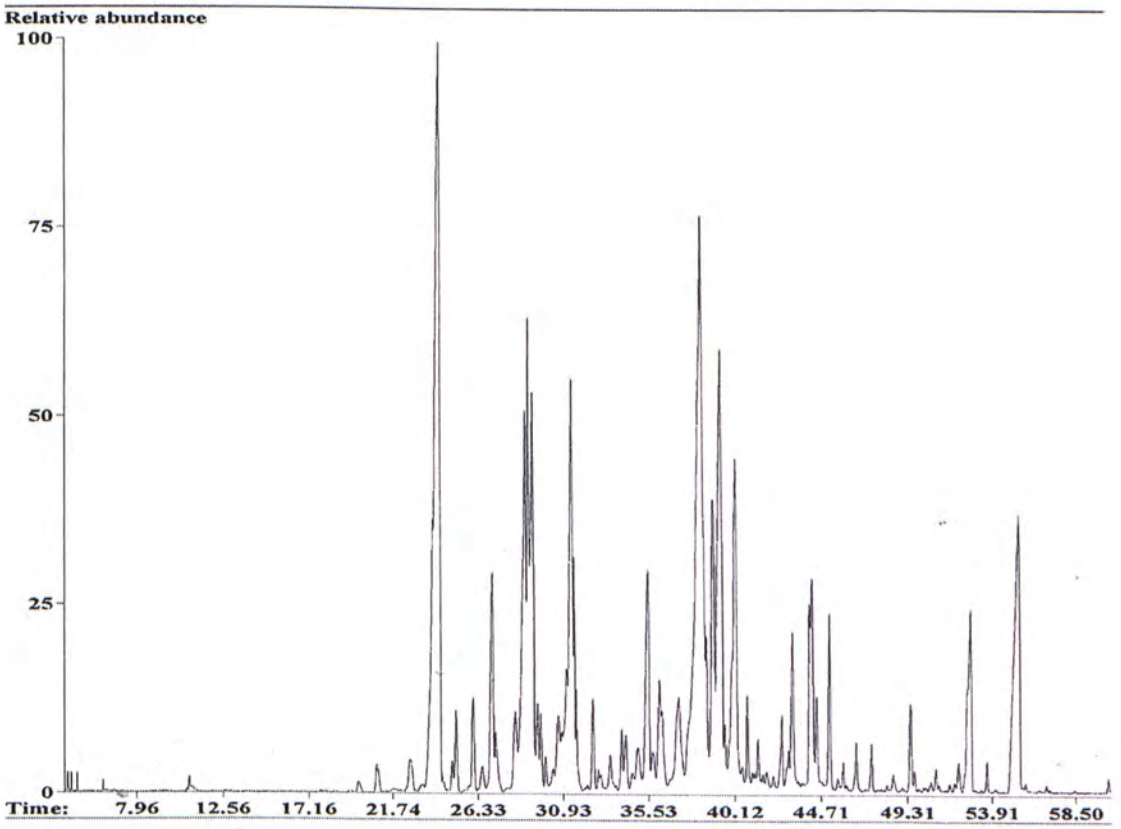

Figure A-37. Gas chromatogram of sample VS18

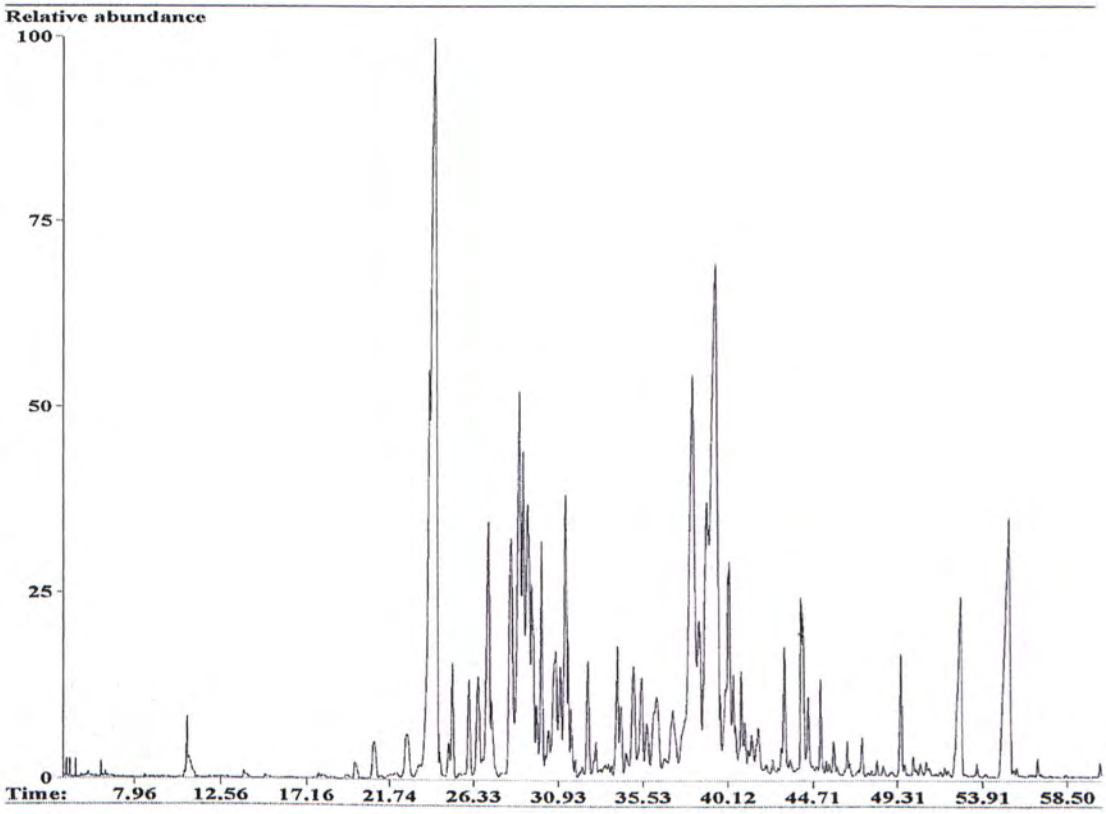

Figure A-38. Gas chromatogram of sample VS19

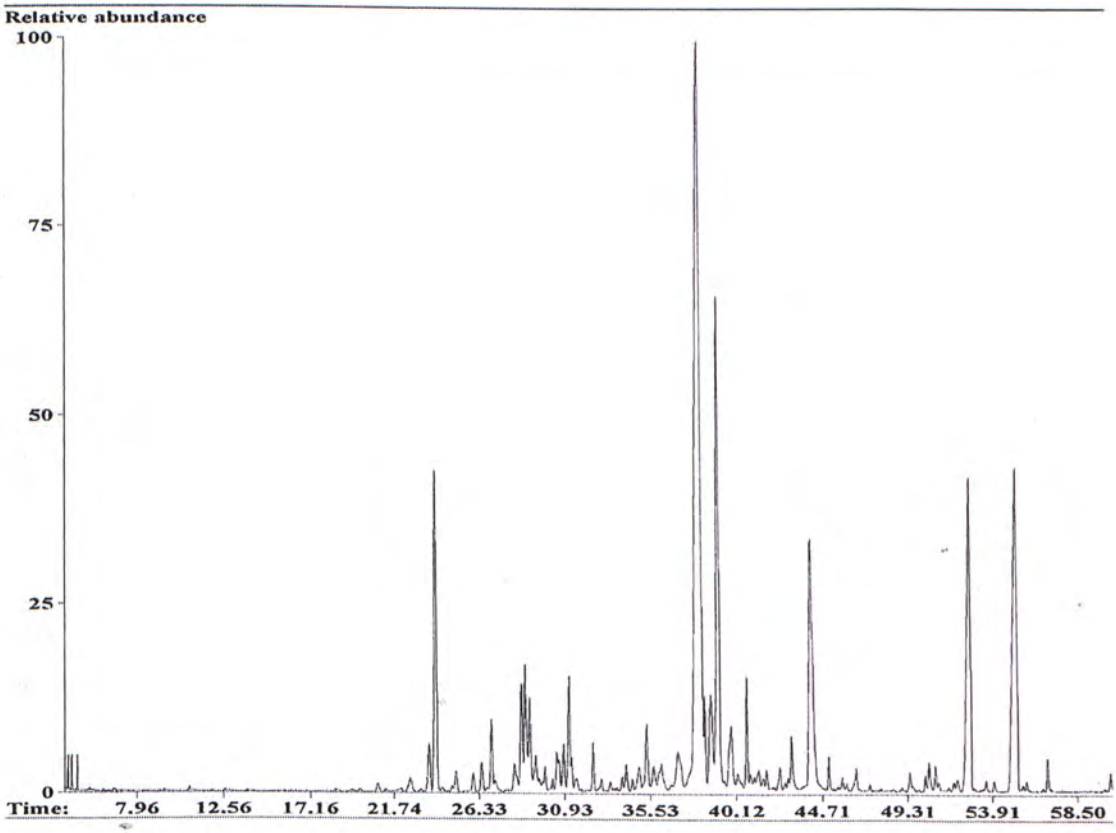

Figure A-39. Gas chromatogram of sample VS20

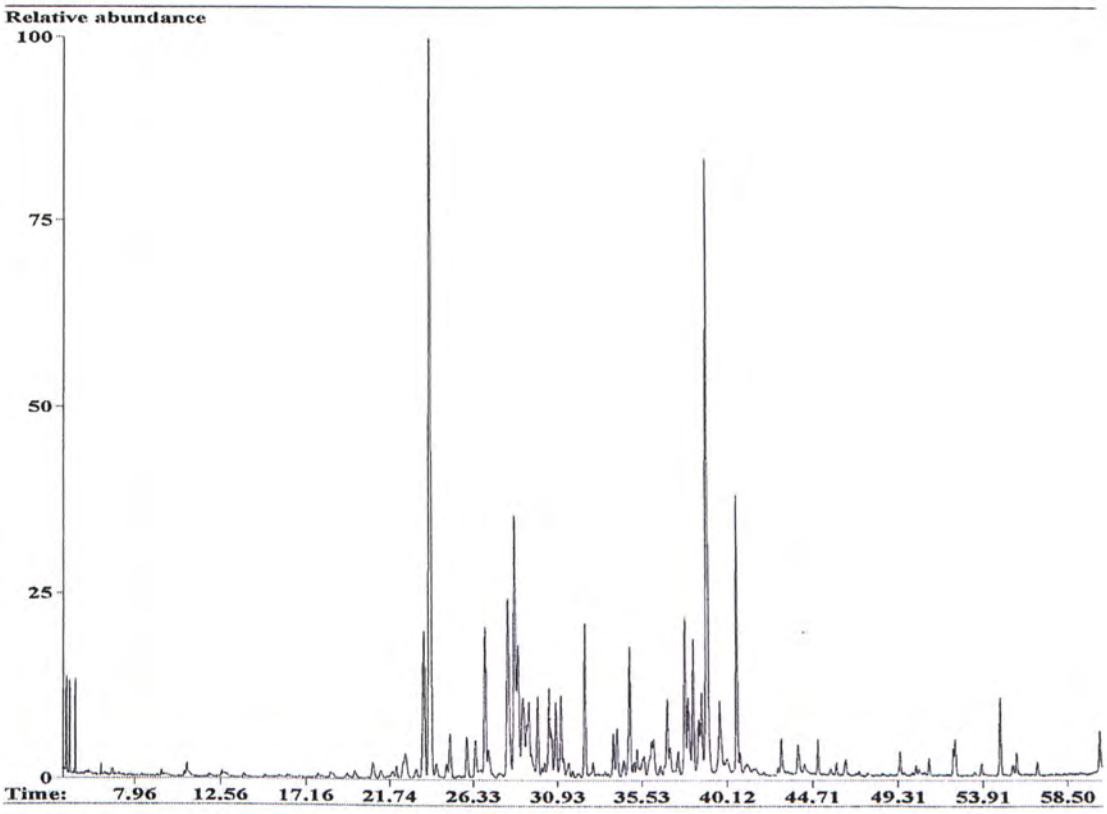

Figure A-40. Gas chromatogram of sample VSV02

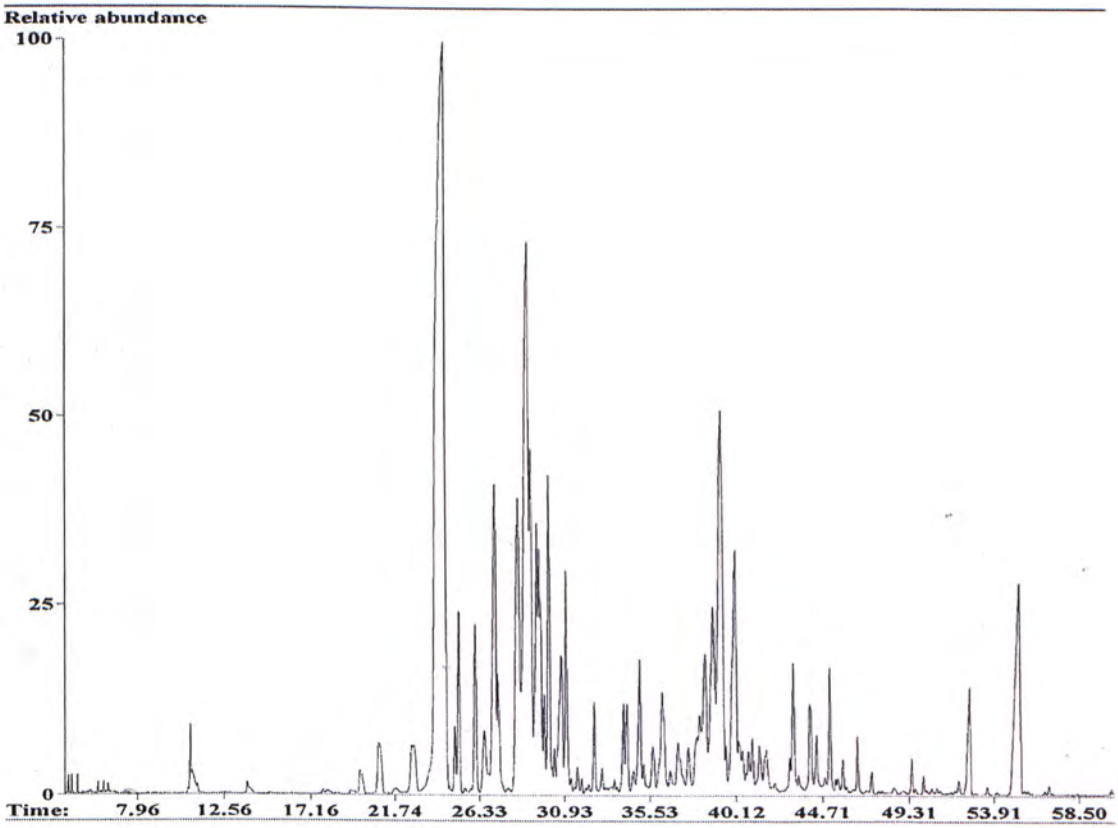

Figure A-41. Gas chromatogram of sample VSV03

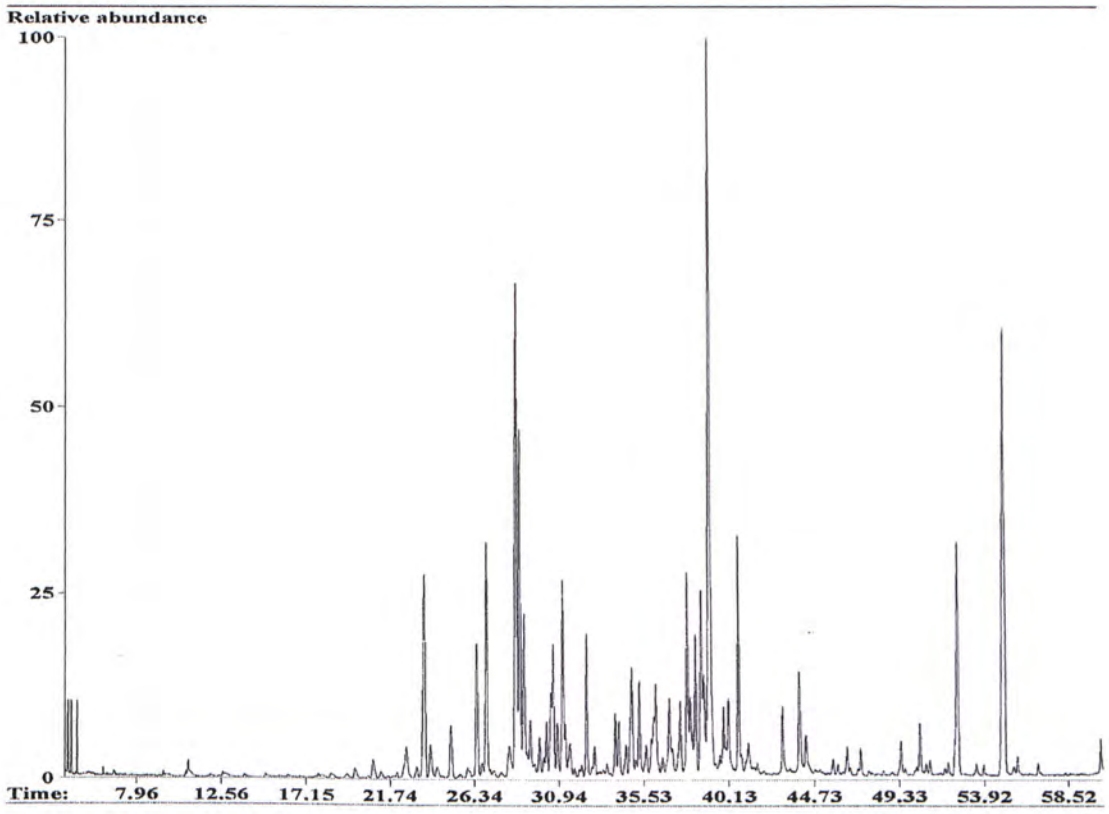

Figure A-42. Gas chromatogram of sample VSV04

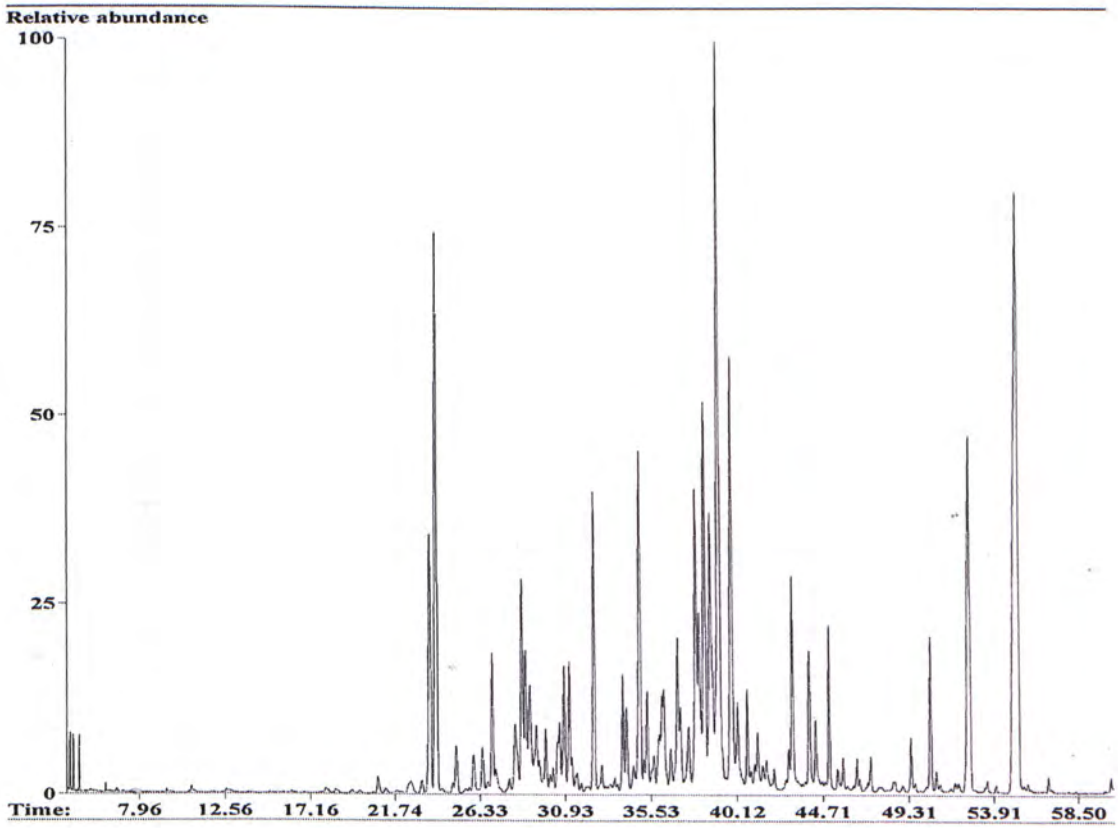

Figure A-43. Gas chromatogram of sample VSV06

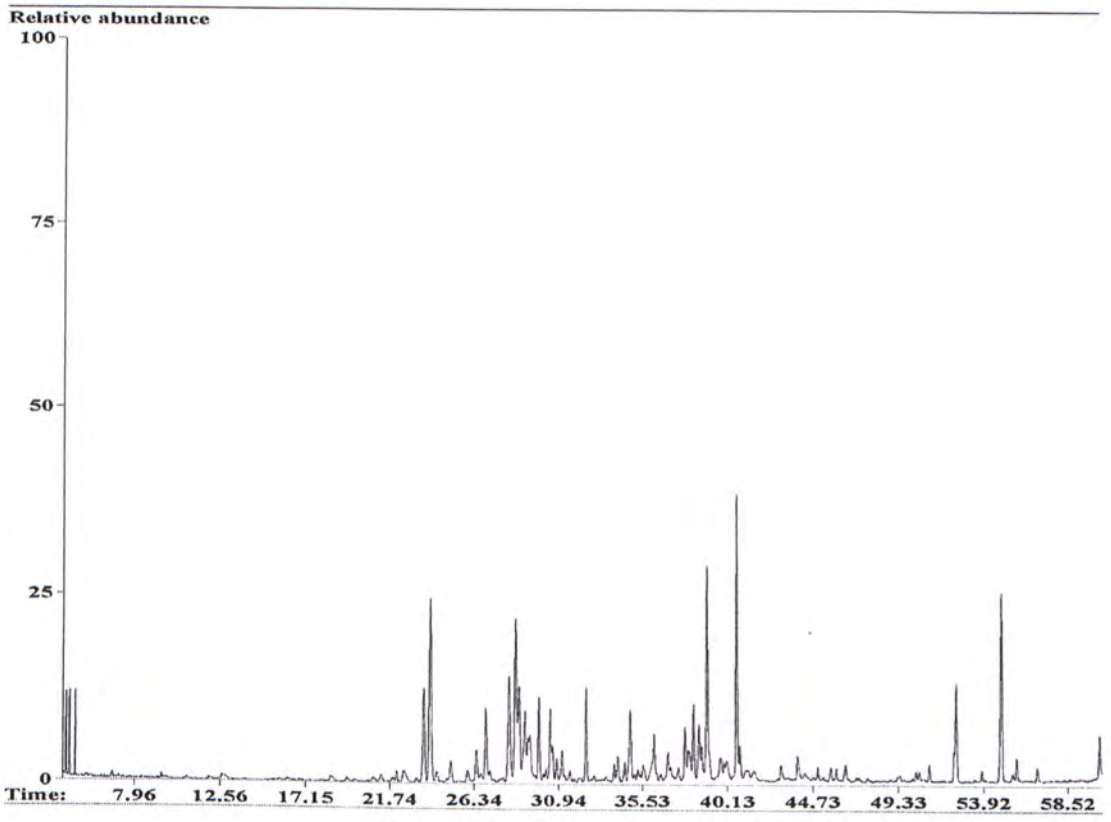

Figure A-44. Gas chromatogram of sample VSV07

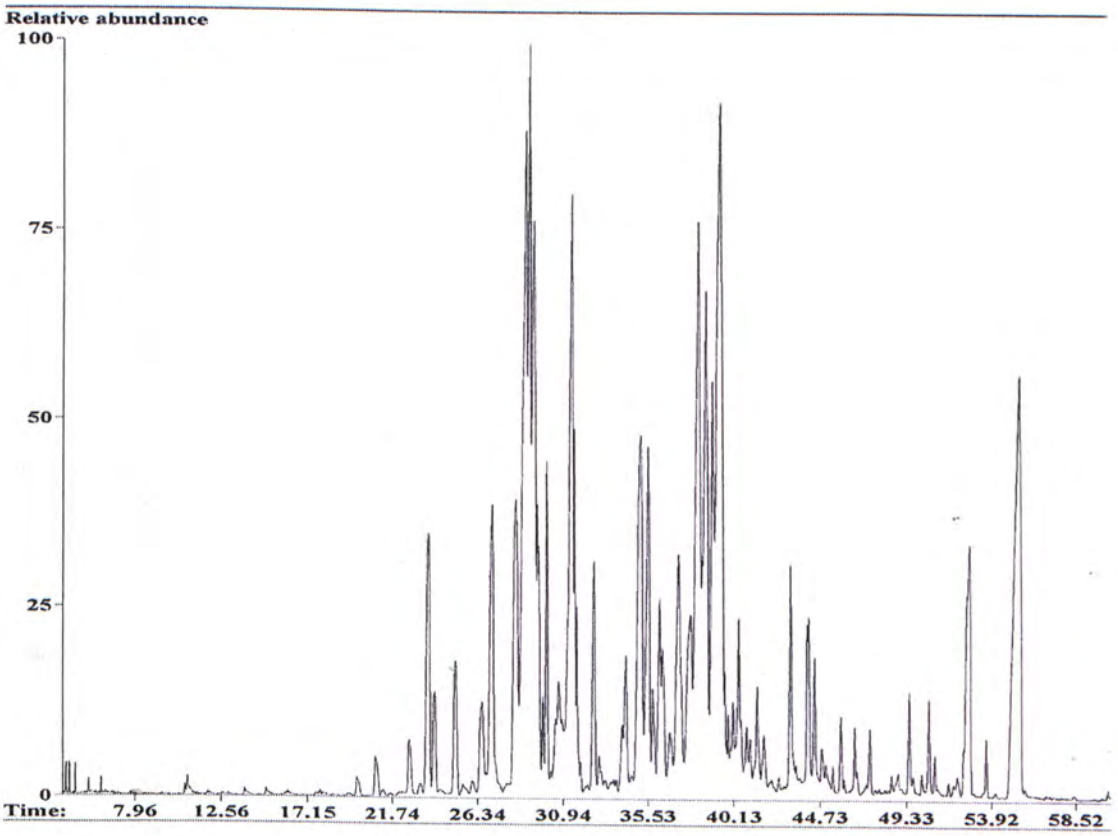

Figure A-45. Gas chromatogram of sample VSV10

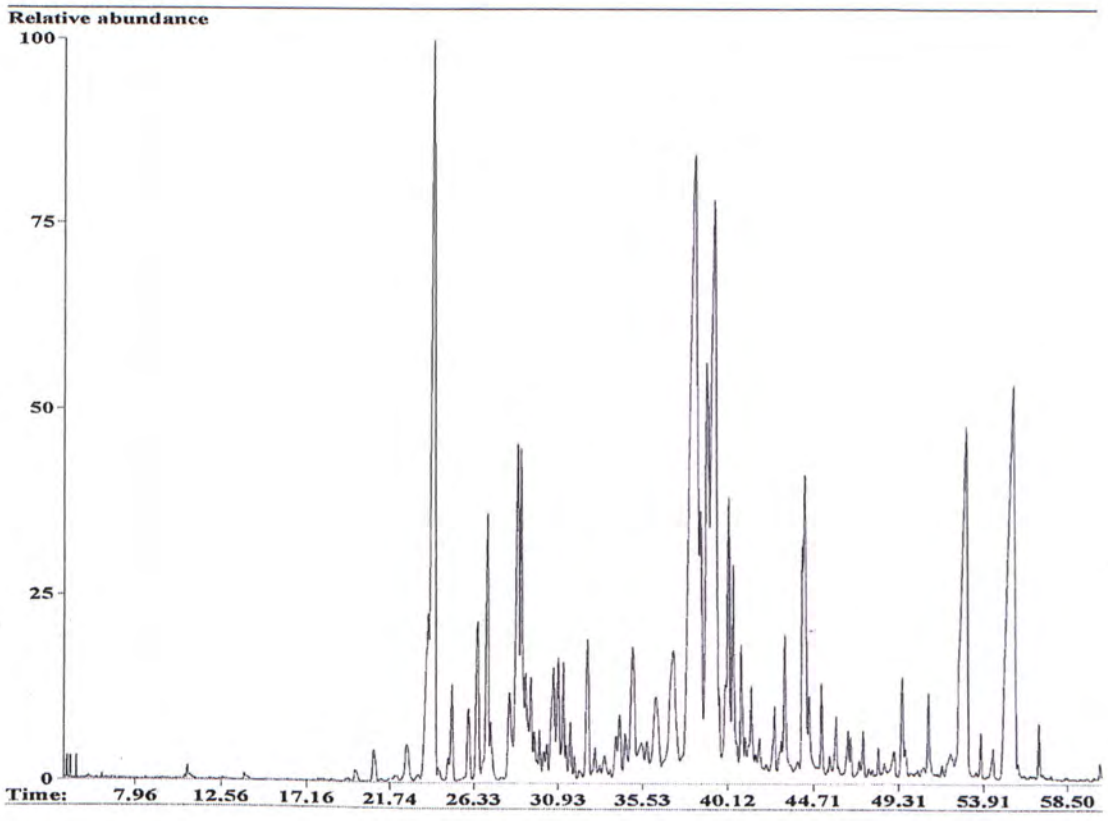

Figure A-46. Gas chromatogram of sample VSV11

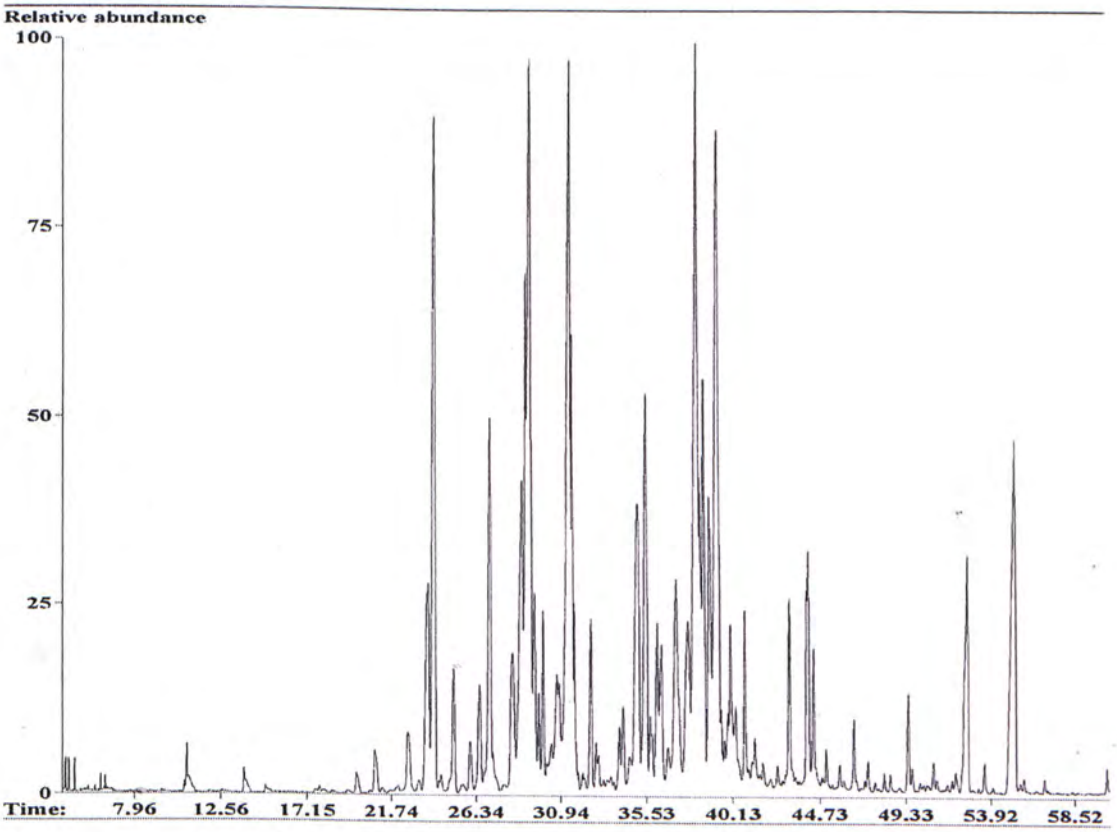

Figure A-47. Gas chromatogram of sample VSV18

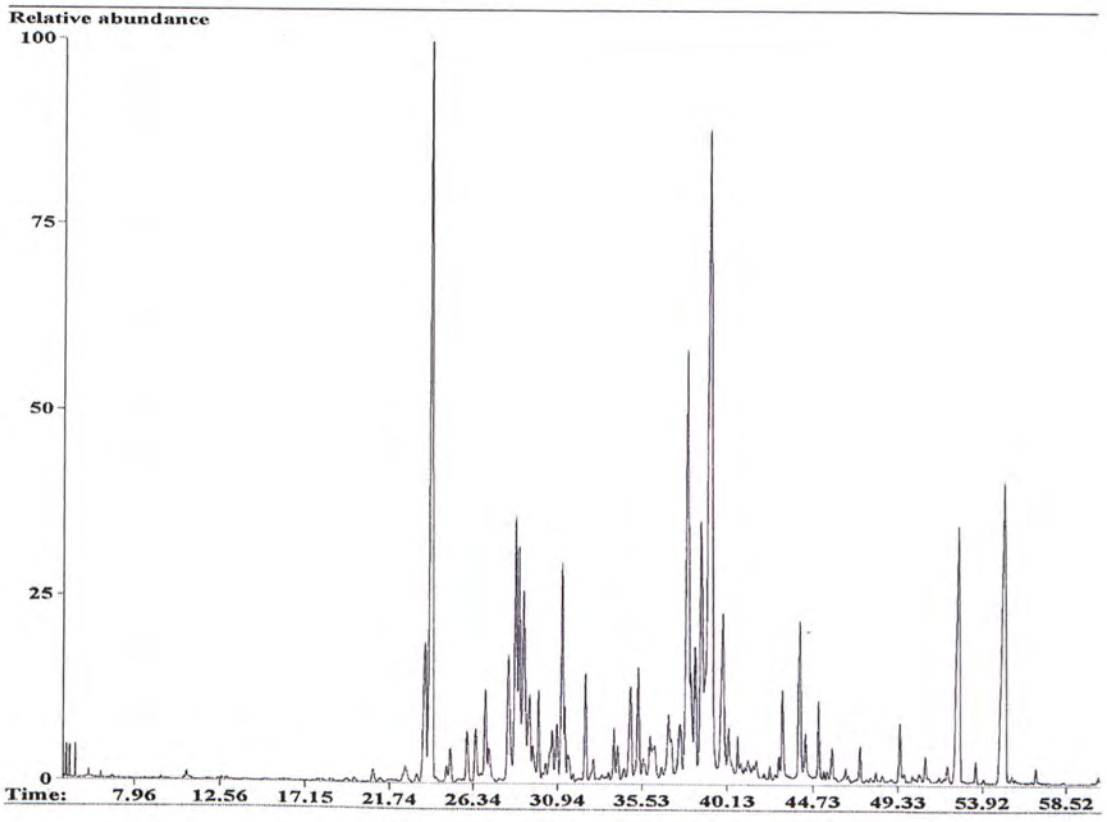

Figure A-48. Gas chromatogram of sample VSV19

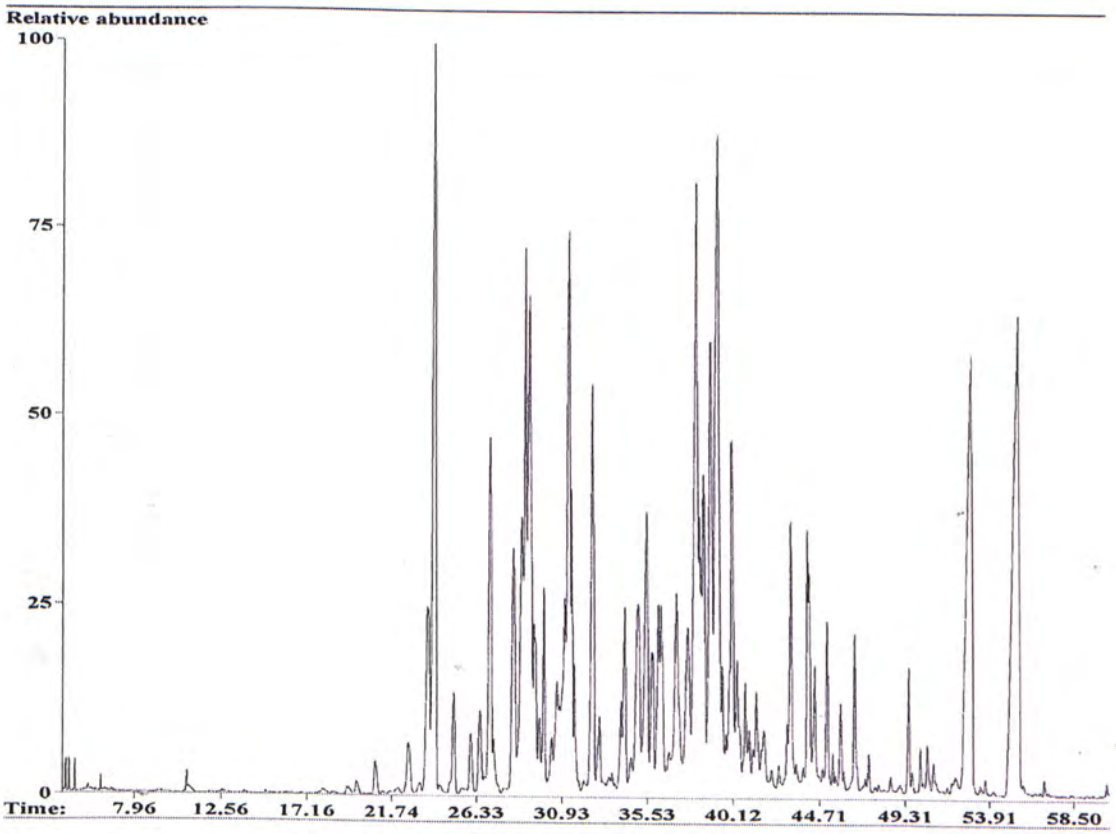

Figure A-49. Gas chromatogram of sample VSV20